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Isolation and characterization of variants of  
polyoma transformed BHK 21 cells defective in  
adhesion to fibronectin.

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A thesis submitted to the University of Glasgow  
for the degree of Doctor of Philosophy.

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## SUMMARY

In order to investigate the interplay between the cell surface and the cytoskeleton in adhesion, variants of polyoma-transformed BHK 21 cells were isolated with or without N—methyl-N-nitro-N-nitrosoguanidine as a mutagen, which are of very low adhesiveness towards surfaces coated with fibronectin. On culture surface the variants yield loosely attached colonies of very distinctive unspread morphology.

Variants retain the ability to cap fluorescent Con A, and are unable to spread on any surface yet tested, including adsorbed serum, Con A, fibronectin, vitronectin or poly-L-lysine. In a flow-chamber adhesion assay, which minimises the contribution of gross spreading to adhesion, the variants are much less adhesive to fibronectin or serum films than the parental cells, but they adhere equally to poly-L-lysine.

A high proportion (35%) of cells in a variant population showed spreading ability on a clean tissue culture plastic surface. The proportion of variants spread is less than that of parental cells. Mn at  $10^{-4}$  M did not stimulate these variants to spread to full extent, but there was an increase in spreading in response to manganese on tissue culture plastic in absence of serum.

An f-actin specific fluorescent probe showed the distribution of f-actin in Py3 cells on a surface coated with fibronectin. Stress fibres appeared very distinct. No stress fibres were observed in the variants in the course of study. f-actin was seen underneath the plasma membrane and at cell-cell contact areas.

Proteins retained in detergent insoluble residues of Py3 and variants were studied by various techniques. No differences in protein bands were seen.

The alteration in these variants can be explained as follows:

1. There are simultaneous changes in the ability of cell surface to bind fibronectin and in the cytoskeletal control.
2. The putative fibronectin receptor, altered in the variant, is involved in some way in the spreading response on all surfaces.

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**Chapter One**  
**INTRODUCTION**

## I. IMPORTANCE OF CELL ADHESION

An understanding of the adhesion of cells of animal tissues in molecular terms could shed light on a number of important biological phenomena such as :

1. Morphogenetic movements : processes occurring during embryogenesis such as gastrulation and the migration of individual cells.

2. Repair (homeostatic) processes, such as thrombosis and wound healing, which play an important part in organ stability, and in the pathology of various disease states.

3. Malignant behaviour: adhesion is thought to be important in local invasion and infiltration, and therefore in metastasis.

4. Artificial surfaces: cell-substratum adhesion is important in tissue culture because cells often need an adhesive surface in order to grow, and in relation to medical prostheses. This may have application to commercial processes.

### 1. Morphogenetic movements

Edelman (1984) has suggested that morphogenetic movements result from the interplay of cellular motility, tension in sheets of tissue and adhesion itself. Weiss (1941) proposed that each molecular contact is specific and like an antigen-antibody link reaction and also he suggested that alterations in the adhesiveness of cells may



be a reflection of changes in the antigenicity of the surfaces. During morphogenetic movements, adhesive contacts must frequently be made or broken. This can be seen for example in gastrulation, and in migration of cells from the neural crest. During amphibian gastrulation, the primary mesenchyme cells break loose from the ectoderm at the grey crescent of the blastula and then crawl over the inner face of the wall of the blastula by putting out contractile pseudopods with adhesive tips which can firmly attach to the epithelium (Trinkaus, 1984). This process clearly involves both cell-substratum (fibronectin) and cell-cell adhesion. In neurulation the folding of the neural plate into a tube may require the preferred adhesion of the neural tube cells with one another and a decrease in adhesion to the mesenchymal cells (Trinkaus, 1984). The subsequent migration of neural crest cells from the neural tube to specific areas of the developing embryo may depend on their separation from each other and adhesion to the surface over which they move (Trinkaus, 1984). Both processes were inhibited when monoclonal antibodies to fibronectin (Boucant et al., 1984 a), or synthetic decapeptides such as (Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro) containing the fibronectin cell-recognition sequence (Boucant et al., 1984 b) were microinjected into early embryos.

## 2. Repair (homeostatic) processes

Cell adhesion is important in maintenance and repair of damaged tissues. In particular cells undergo transitions

from suspended (circulating) to adherent, such as lymphocytes during recirculation, platelets in blood clotting, and neutrophil leucocytes in the response to inflammation. In lymphocyte recirculation, the cell must stick to the endothelium of the capillary venule (Bell, 1978) in face of the strong shearing force of blood flow, and crawl into the lymphoid tissues. Similarly, when a blood vessel is damaged, platelets escape from the blood stream through the wound, then adhere to collagen fibres in the connective tissue. Secretions such as the hormone serotonin, protein and ADP then stimulate aggregation of platelets with one another and adhesion to the fibrin network forming at the site of injury to the endothelial lining of the vessel, in such a way as to plug the defect (Gordon, 1980).

During the acute inflammatory response, neutrophil granulocytes (PMNs) initially adhere to the fibronectin-deficient laminar surface of endothelium, move over the surface of the endothelial cells towards an endothelial cell junction, and migrate through the matrix of the tissue (which contains fibronectin and collagen) towards inflammatory foci. Upon reaching the lesion, neutrophils phagocytose the pathogen and necrotic tissue, and release a wide range of hydrolytic enzymes and possibly factors which attract other neutrophils (Rayan et al., 1977).

Some of these circulating cells originate from adherent cells and so show the reverse of the above transitions. For example the release of erythroid cells

from bone marrow into the circulation is accompanied by the loss or modification of their cell surface fibronectin-binding sites (Patel et al.,1985).

An example of adhesion in a repair process which does not involved suspended cells can be seen in *Xenopus* tadpoles (Trinkaus,1984). During wound healing, the basal layer moves over the basement membrane, and carries the outer layer on its back. This involves adhesion as follows: the active spreading basal layer adheres to basal membrane and the outer layer adheres by means of desmosomes to the basal layer ( Radice,1980).

### 3. Malignant behaviour

The absence of normal constraints on the locomotory ability of many neoplastic cells can result in the invasion of surrounding tissue and the metastatic spread of malignant cells to other parts of the body. The passive dissemination of cancer cells and the subsequent establishment of secondary tumours may involve changes in both their adhesiveness and motility.

In order to form a successful secondary, tumour cells must first detach from primary tumour. A decreased mutual adhesiveness would obviously facilitate this process. Differences in the adhesion strength between normal and tumour cells were first observed by Coman (1961) who measured the force required to separate cells. This showed that adhesion was much weaker in carcinoma cells than

epithelial cells, but various subsequent studies with cells transformed in vitro have not yet yielded a coherent picture. In some cases, the adhesiveness of transformed cells relative to their normal counterparts was reduced (Edwards et al., 1977; Tanaka et al., 1980; Whur et al., 1977; Dennis et al., 1982; Brackenbury et al., 1984), whereas in other cases it was increased (Halpern et al., 1966; Wright et al., 1977 & 1981) or no appreciable changes were found (Winkelhake & Nilson, 1976)

#### 4. Artificial surfaces

The interaction between cells and non-physiological surfaces is very important for a variety of reasons: first, many kinds of tissue cells are grown in culture on artificial surfaces. The majority of cells derived from solid tissues, provided they are not transformed, are unable to grow suspended in a fluid medium. They must be provided with a solid surface to which they can adhere and on which they spread. These cells are described as anchorage-dependent (Shin et al., 1975). Cells which have undergone transformation frequently become anchorage-independent and can grow in suspension. Second, adhesion of cells to non-physiological substrates is important in medical applications, especially in relation to prostheses, where non-adhesive surfaces for integration with tissue are used, for example tantalum alloy for bone prostheses.

## II. IDENTIFICATION OF PLASMA-MEMBRANE MOLECULES INVOLVED IN ADHESION

Cell-to-cell interactions, such as adhesions and exertions of force by or upon cells, are carried out through the plasma membrane and the molecules composing it and associated with it. Therefore it has been considered that adhesion depends either on:

1. Non-specific molecular interactions, such as the long range attractive force interacting with electrostatic repulsion (Curtis, 1960).
2. The close-range interaction properties of specific cell-adhesion molecules (Moscona, 1968; Edelman, 1984 a).
3. A combination of 1 and 2.

Workers favouring the second type of mechanism have isolated a series of such cell adhesion molecules, probably intrinsic glycoproteins of plasma membranes. There are two different phenomena which have been investigated: the adhesion of cells to each other, and the adhesion of cells to extracellular substrates.

### A. Cell-cell adhesion

There have been several different approaches used in the identification of molecules that may be involved directly or indirectly in cell-cell adhesion. The three main approaches employed have been as follows:

1. The isolation and identification of the components of intercellular junctions.
2. The production of antisera to cell surface molecules, leading to the use of monovalent antibodies in the inhibition of cell-cell adhesion. This has also been used in relation to cell-substratum adhesion (see section B below).
3. Examination of conditions favouring or breaking adhesion.

During embryogenesis, once cells have made definite contacts with the cells with which they will form tissues and organs, the plasma membranes of neighbouring cells in many cases quickly establish structural specializations in the form of cell junctions or appositions, in which the opposed cell surfaces have standard and reproducible relation to each other.

In many instances cells may adhere to one another by simple membrane appositions in which the plasma membranes are separated by electron-lucent spaces of about 20 nm and no specializations are seen. However morphological studies of specialized cell junctions are of importance when considering the mechanisms of cell adhesion. Electron microscope studies have revealed several different types of junctions (see Trinkaus, 1984). The main types are: tight junctions or zonulae occludens, desmosomes or maculae adhaerentes, intermediate junctions, and gap junctions.

In the tight junction (zonula occludens), the opposing plasma membranes appear to be in direct contact. From the work of Farquar & Palade (1963) junctions of this type would appear to encircle cells in some epithelia. The adjacent plasma membranes are held together by continuous strands of junctional proteins that make contact across the intercellular space or by direct contact of the lipids.

In the maculae adhaerentes, plasma membranes are 20 nm apart. The gap between is filled with slight staining material. Tonofilaments, within the cytoplasm are associated with this junction.

In the intermediate junctions (originally named zonulae adhaerentes, Farquhar & Palade, 1963) contractile bundles of actin filaments run along the "belt desmosomes". These filament bundles probably mediate one of the most fundamental processes in morphogenesis, the folding of the epithelial cell sheet into tubes (Staehelin et al., 1978). There are similarities between intermediate junctions and the focal adhesions formed between cells and culture substrates, where  $\alpha$ -actinin and vinculin are concentrated in attachment plaques (Geiger, 1979; Burridge & Feramisco, 1980) (see below).

The gap junction appears to have an intercellular separation of 2 nm. Freeze cleave preparations of cells reveal a hexagonal array of subunits (Goodenough & Revel, 1970). Following Lowenstein (1968) much subsequent

work has confirmed that these junctions are areas of electrical coupling between cells. Possibly they also contribute to intercellular adhesion.

The diversity of morphological contacts observed by electron microscopy suggests there may be more than one adhesive mechanism involved in cell-to-cell adhesion. It has been reported that the major or perhaps only function of desmosomes is in providing a structural basis for cell-cell adhesion in epithelia, whereas zonular tight junctions seal the space between cells in epithelia, thus making the sheet impermeable, and possibly also contributing a barrier to diffusion of intrinsic membrane proteins.

The elucidation of the molecular interactions responsible for cell-to-cell adhesion and for filament attachment in the various junctions requires isolation and identification of adhesion molecules.

Desmosomes were first isolated from bovine nose (Skerrow & Matoltsy, 1974 a, 1974 b; Skerrow & Skerrow, 1980), and their proteins separated into more than 20 polypeptides of molecular weights 15 Kd -230 Kd in SDS-PAGE gels. The function of the various components was not then determined and the mechanism of adhesion between the two halves of the desmosomes is (and remains) unknown. However they found several periodic Schiff's reagent (PAS)-positive polypeptides with molecular weights in the region 120 Kd-140 Kd which constituted 23% of the desmosomal



protein. The authors suggested that these glycoproteins are involved in cell-cell adhesion at the desmosomal junction. In support of this, it was observed that extraction of desmosomes with EDTA removes some of these glycoproteins and causes widening of the intercellular gap (Skerrow & Skerrow, 1980; Gorbsky & Steinberg, 1981) they have also reported the presence of glycoproteins of molecular weights 100 Kd, 115 Kd and 150 Kd in desmosomes of bovine muzzle epidermis. Presumably there were discussing the same glycoproteins detected by Skerrow et al. (1974 a, b, & 1980).

Cowin et al. (1984) used specific antibodies to determine the intra- or inter-cellular location of desmosomal components, and to identify possible desmosomal adhesion molecules. Antibodies against the desmosomal glycoproteins of molecular weight 150 Kd, 115 Kd and 100 Kd, react with surfaces of impermeabilized and live MDBK cells, and also the authors found that Fab' fragments derived from these antibodies inhibit desmosome formation in culture. They suggested that these molecules are located on the cell surface and are directly involved in intercellular adhesion.

#### Identification of cell-to-cell adhesion molecules by antibodies

The use of inhibitory univalent antibodies to identify molecules involved in cell-adhesion was applied to the cellular slime mould Dictyostelium discoideum by

Gerisch (1980). The adhesive species, "contact-sites", could be defined by their ability to neutralize the aggregation-inhibiting activity of Fab. This work led to the isolation and characterization of a plasma membrane glycoprotein almost certainly responsible for intercellular adhesion in these organisms and to the successful extension of the approach to a variety of cells.

Using this approach, Edelman and colleagues have identified and characterized a series of cell adhesion molecules (CAMs) from chick embryos, N-CAM, L-CAM and Ng-CAM (Edelman et al., 1984).

#### Neural cell adhesion molecule (N-CAM)

Brackenbury et al. (1977) used inhibitory Fab to investigate the mechanism of adhesion between cells of the chick embryonic neural retina. Immunoglobulin from rabbits immunized with embryonic retinal cells agglutinated the cells in suspension, but its Fab inhibited aggregation dramatically. Three polypeptides which could neutralize this Fab were isolated from culture supernatants (serum-free) using gel filtration and preparative gel-electrophoresis, with molecular weights 140 Kd, 120 Kd and 65 Kd. Antibodies raised against the purified components precipitated a 140K dalton species from detergent solubilized membranes, which has since been named neural cell adhesion molecule (N-CAM) (Thiery et al., 1977).

The sialic acid content of N-CAM varies in different

tissues (Rothbard et al., 1982) as well as with the developmental age (Edelman & Chuong, 1982).

Grumet & Edelman (1984 ) found that the adhesion between brain vesicles and cells was inhibited when specific antineural Fab' fragments were used, and also that when antineural Fab' fragments were microinjected into the tectum of Xenopus tadpoles, retinotectal projections were disrupted (Frazer et al., 1984).

Chuong et al. (1982) have found a similar neural adhesion molecule exists in the mouse, named mouse N-CAM, and have confirmed that it is also present in rat neural tissue. The similarity is based on both functional analogy and structure. Mouse N-CAM and chicken N-CAM cross-react immunologically, amino acid compositions and high sialic acid contents of the molecules are similar. The authors suggested that the mouse N-CAM is evolutionarily related to chicken N-CAM and prompt the hypothesis that cell adhesion involving N-CAM is a fundamental mechanism existing in nervous systems of different vertebrate species.

Another cell adhesion molecule has been identified from rat neuronal plasma membrane. Jorgensen et al. (1980) and Hirn et al. (1983) found three polypeptides, in foetal brain one of molecular weight 200 Kd- 250 Kd, and a second of 140 Kd, the third, 130 Kd in adult brain. These have been named D2-cell adhesion molecule (D2-CAM) (Jorgensen et

al.,1980; Hirn et al.,1983). This adhesion molecule (D2-CAM) has been shown to cross react immunologically with chicken N-CAM (Jorsensen et al.,1980; Edelman,1983) and with a brain specific protein (BSP-2) (Hirn et al.,1983). These authors reported that D2-CAM from rat displays several similarities to chicken N-CAM such as the molecular weight 139 Kd and 140 Kd. The authors suggested that N-CAM from chick embryo and D2-CAM from rat are related molecules occurring in different species.

The authors reported that D2-CAM may be involved in neurite-neurite interaction, a property which has been reported for cell adhesion molecule (CAM) isolated from chick embryo neural tissue, when Fab was found to inhibit sorting out of cell bodies and neurites and to decrease the number of membrane-membrane contacts (Thiery et al.,1977; Rutishauser et al.,1978). Its mode of action may be modified during development in the relative synthesis of the different polypeptides, as well as by changes in their glycosylation and sulfation (Lyles et al.,1984).

#### Neuron-glia cell adhesion molecule (Ng-CAM)

A cell adhesion molecule (Ng-CAM) has been identified on neurons (Grumet et al.,1984 a) by using monoclonal antibodies, which mediates the heterotypic adhesion between neuronal and glial cells (Thiery et al.,1985). It has been found that Ng-CAM and N-CAM, both of which have calcium-independent binding mechanisms, have

different functions in the nervous system, as Fab fragments of antibodies to N-CAM did not prevent the binding of neurons to glial cells (Grumet & Edelman 1984).

#### Liver adhesion molecule (L-CAM)

The use of inhibitory Fab also led to identification of a cell adhesion molecule of molecular weight 124 Kd from chick embryonic liver, named liver cell-adhesion molecule (L-CAM) (Bertolotti et al., 1980; Gallin et al., 1983). Liver cell adhesion molecule (L-CAM) is a calcium-dependent cell adhesion molecule found in very early vertebrate embryos and on liver and other epithelial cells in adults (Gallin et al., 1985).

The use of Fab preparations from rabbit antisera against mouse embryonic carcinoma cells has led to the isolation of an 84 Kd fragment of a 120 Kd cell surface glycoprotein, uvomorulin,. This adhesion molecule (UM) appears to exist at the cell surface of embryonic carcinoma cells as a 120 Kd molecule that can be degraded into a variety of fragments (Peyrieras et al., 1983). A fragment of the 120 Kd glycoprotein was purified by gel filtration and chromatography on ConA- Sepharose to yield a glycoprotein of molecular weight 84 Kd in SDS-PAGE. UM appears to be responsible for the compaction of cleavage stage mouse embryos (Hyafil et al., 1980). This 84 Kd fragment was the major glycoprotein released by trypsin treatment of embryonal carcinoma membranes. UM seems similar to L-CAM, especially in view of the observations of Gallin et

al.(1983 ) that an antigenically active L-CAM derivative can be released from the cell surface as a fragment of molecular weight 81 Kd by proteolysis with trypsin in the presence of calcium. However Edelman (1984 a) believes that L-CAM, 120 Kd, is an intrinsic glycoprotein, whereas Peyrieras et al.(1983) gave evidence that UM lacks a membrane inserting hydrophobic sequence.

#### L-CAM and calcium-dependence

Hyafil et al.(1981) found that  $\text{Ca}^{2+}$  protects the 84 Kd glycoprotein tryptic fragment against further degradation. This suggests that  $\text{Ca}^{2+}$  alters the conformation of this glycoprotein from a trypsin-sensitive to a trypsin-resistant conformation. This interpretation was supported by the finding that a monoclonal antibody raised against this glycoprotein precipitated the antigen in the presence of  $\text{Ca}^{2+}$ .

Brackenbury et al.(1981) used specific antibodies against N-CAM and L-CAM to investigate the relation between these cell-adhesion molecules and the  $\text{Ca}^{2+}$ -dependent mechanism of cell adhesion. They found that  $\text{Ca}^{2+}$  protected,  $\text{Ca}^{2+}$ -dependent adhesion of neural cells was not inhibited by anti-(N-CAM) Fab, but could be inhibited by polyspecific Fab from rabbits immunized against neural cells dispersed by trypsin in the presence of  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$ -independent aggregation was dependent on N-CAM. L-CAM-dependent aggregation was  $\text{Ca}^{2+}$ -dependent, but was not

inhibited by the Fab active against the neural  $\text{Ca}^{2+}$  dependent mechanism.

Takeichi et al. (1981) have investigated the  $\text{Ca}^{2+}$ -dependent aggregation of embryonal carcinoma (EC) cells. Pronase has to be used to disperse these cells, but even with this enzyme,  $\text{Ca}^{2+}$  was found to protect a  $\text{Ca}^{2+}$ -dependent adhesive mechanism.

Uvomorulin has also been found to be involved in a  $\text{Ca}^{2+}$ -dependent form of intercellular adhesion and is itself protected against tryptic degradation by  $\text{Ca}^{2+}$  ions. There could be a parallel with findings of Urushihara et al. (1977) for BHK 21 cells and Chinese hamster V79 lung cells (Takeichi, 1977) and for chick embryonic neural retina (Takeichi et al., 1979) and embryonal carcinoma cells (Takeichi et al., 1981) that each could exhibit  $\text{Ca}^{2+}$ -dependent aggregation provided  $\text{Ca}^{2+}$  was present during their proteolytic dispersal.

#### Structure of Known CAMs

The topography of L-CAM resembles that of N-CAM, upon proteolysis both L-CAM and N-CAM release a fragment from the cell surface, of about two-thirds of the molecule. The fragments include the amino-terminal portion of the polypeptide chain, the bulk of the carbohydrate, and the antigenic sites recognized by antibodies that block cell adhesion (Cunningham et al., 1984). Both CAMs have phosphothreonine and phosphoserine residues in the

carboxyl-terminal third of their peptides (Cunningham et al., 1984). However, the polypeptides and fragments produced from them by proteolysis were found to differ in size, and the molecules have different amino-terminal sequences (Cunningham et al., 1984 ) and are coded by different genes (Edelman, 1984).

N-CAM, 170 Kd, appears to be oriented with the amino terminus extending away from the cell surface, includes an N-CAM to N-CAM binding site (fragment of 65K) and has the bulk of its sialic acid near the middle of the peptide chain (fragment of 108 Kd). The carboxyl-terminal region includes the portion of the polypeptide chain that inserts in the plasma membrane (Cunningham et al., 1983 ).

Edelman (1984 a) reported that N-CAM and L-CAM are segregated into different tissues during gastrulation and early neurogenesis, and are part of a regulated genetic program during development (Gallin et al., 1985). During development there is a sequence of both reversible and irreversible expression of CAM genes coordinated with epigenetic alterations in the surface expression of different CAMs.

Edelman also (1984 b) proposed a regulator hypothesis "a sequence for the genetic and molecular regulation of morphogenesis" which is intended to provide a specific molecular framework relating developmental genetics to



evolution. The hypothesis derives from an analysis of the interactive morphogenetic roles of the primary processes of cell adhesion.

## B. Cell-matrix attachment

As well as adhering to one another, cells also attach firmly and spread out on or in inert matrices of both biological and artificial origin.

Microscopic observations (anatomic, histological studies) have revealed that many cells adhere to extracellular matrix in vivo. In vitro, adhesion to a culture substratum is often a prerequisite for biosynthesis of macromolecules and growth of mesenchymal and epithelial cells (Folkman & Moscoma, 1978; Ben-Ze'ev et al., 1980). Such adhesion, which mimics in vivo anchorage to extracellular matrix, is a multi-step membrane process involving recognition of extracellular adhesive molecules such as collagen (various types), fibronectin, laminin, and vitronectin by cell surface receptors, subsequently followed by the reorganization of the cytoskeleton that leads to cell spreading (Grinnell, 1978; Hynes, 1981).

### 1. Morphology of cell-substratum adhesion

Fibroblasts cultured on plane substrates appear to adhere to such surfaces by two distinct types of contact involving discrete areas of their ventral surfaces: focal contacts, which are small punctate regions of closest approach (10-15 nm); and close contacts, broader areas, often surrounding focal contacts, with spacing around 30 nm (Izzard & Lochner, 1976). These areas can be detected in the

light microscope as black and grey areas respectively, using interference reflection microscopy.

Electron microscopy (Abercrombie et al., 1971) and immunofluorescence (Geiger, 1979) have shown that the cytoplasmic faces of focal adhesions are sites where actin-containing stress-fibres terminate. Focal adhesions usually occupy a relatively small proportion of the ventral surface area of a cell.

Vinculin, identified by Geiger (1979), was found to match focal adhesions closely. This cytoskeletal protein of molecular weight 130 Kd, was found as a contaminant during the purification of  $\alpha$ -actinin from the smooth muscle of chick gizzard, heart and skin cells. Vinculin was localized in arrow-head foci at the edge of leading lamellae and in short streaks further from the periphery. Vinculin has also been found associated with regions of attachment of microfilament bundles to plasma membranes in other structures such as intercalated disc of the cardiac muscle (Geiger et al., 1980).

Attempts to identify cell surface molecules associated with focal adhesions have been less successful. Because fibronectin is active in promoting adhesion and spreading of cells on artificial surfaces, fibronectin fibrils are found in close registry with microfilament bundles. Chen and Singer (1980) used a double-labelling technique to locate fibronectin and Con A binding proteins

in ultrathin frozen sections of embryonic chick heart fibroblasts. Labelled antibody to fibronectin appeared to be specifically excluded from both focal and close contacts.

Badley et al. (1980) observed a lack of correlation between fibronectin fibrils and focal adhesions in 16 C rat dermal fibroblast cells. The authors also examined the distribution of staining with a fluorescent conjugate of the galactosyl-binding lectin, ricin. They found that the ricin receptors appeared to be present at focal adhesions, but also extended further towards the nucleus in registry with microfilament bundles. The possible importance of carbohydrate in focal adhesion is indicated by the observation that focal adhesions, but not close contacts, seem to be defective in the 3T3 variant AD6. It seems that despite the considerable interest in these structures, intrinsic membrane proteins presumed to be associated with focal adhesions are as yet unidentified.

## 2. Fibronectin

Since the very extensive literature surrounding collagen and fibronectin has been frequently reviewed, I will deal briefly with the matrix molecules themselves, and give more detailed attention to receptors.

Fibronectin is a major glycoprotein of the cell surface of fibroblasts in culture, and of extracellular

matrix found in vivo, that mediates attachment, spreading, motility, and long term survival in vitro of many types of cells when adsorbed to tissue culture substrata (Laterra et al.,1983), and is well characterized in terms of structure (Hynes & Yamada,1982) and mechanism of action (Yamada, Hayashi & Akiyama, 1982).

Many studies indicate that the cellular and soluble plasma forms of fibronectin are structurally and functionally similar, but not identical (Hynes & Yamada, 1982). Fibronectins are synthesized by a wide variety of cells. The differences between cellular and plasma fibronectins could arise from the existence of multiple genes, from alternative processing of the primary transcript of a single gene, from posttranslational cleavages, or from some combination of these mechanisms (Schwarzbauer et al.,1985).

It has been found that a single fibronectin gene can give rise to several different mRNAs by alternative splicing (Schwarzbauer et al.,1985). The alternative splicing events occur within the coding region, yielding mRNAs which differ in sequence.

Fibronectin consists of dimeric subunits (Yamada et al.,1977) and has the potential to interact with several cell surface or matrix-associated macromolecules including collagen (Engvall et al.,1978), both fibrin and fibrinogen (Grinnell, 1980), certain glycosaminoglycans (Laterra

et al.,1980; Yamada et al.,1980) and possibly gangliosides (Kleinman et al.,1979), and in particular with a cell-surface receptor involved in adhesion and spreading of cells, presumably the 140 Kd glycoprotein identified by Pytela et al.(1985a)( see below).

### 3. Fibronectin receptor

Conventional binding and saturation experiments failed to identify a fibronectin receptor on cells. Subsequently, different experimental approaches were adopted to try to identify such a receptor such as: cross-linking between cell membranes and derivatized fibronectin (Aplin et al.,1980), inhibition of cell attachment by gangliosides (Kleinman et al.,1979), preparation of antisera or monoclonal antibodies which inhibit cell attachment (Tarone et al.,1980), and affinity chromatography on immobilized fibronectin ( Yamada & Kennedy, 1984).

#### Cross-linking

Aplin et al. (1980) used reversible cross-linking experiments in which derivatized fibronectin acted as a spreading factor for attached BHK 21 cells. Plasma fibronectin was first covalently coupled to glass coverslips and the attached protein derivatized with a cleavable photoreacting cross-linking agent. The coupling reactions did not affect the activity of the fibronectin for cell attachment and spreading. Cellular components

trapped by cross-linking were then released by reductive cleavage and analyzed by SDS-PAGE.

These experiments showed that a glycoprotein with a molecular weight of 47 Kd was in close proximity to the substratum-bound fibronectin in adherent BHK-21 cells. Some material of very high molecular weight, possibly proteoglycan was also observed. Fibronectin seems to bind predominantly to pre-existing extracellular matrix, (see below), but there was no trace of specifically bound lipid or glycolipid when cells were labelled with <sup>14</sup>C palmitate. In further experiments where fibronectin was replaced by the lectins Con A, ricin, or soy-bean agglutinin, the same 47 Kd species became uniquely linked as for fibronectin, despite the known ability of these lectins to bind to many different glycoproteins of BHK-21 cells ( Rauvala et al., 1981).

Aplin et al. (1981) suggested that the 47 Kd glycoprotein may become specifically enriched at points of cell-adhesion to the substratum. This raises the possibility that it may become linked to fibronectin for the same reason, rather than having any specific affinity for fibronectin. This idea is given further support by the finding that Fab to the 47 Kd glycoprotein does not cause extensive rounding of BHK-21 cells on Fn-coated substratum ( Hughes, Butters & Aplin, 1981). The 47 Kd glycoprotein described by Aplin et al. (1981) was put forward as a possible candidate for a transmembrane constituent

mediating interactions between the matrix molecules and cytoskeletal proteins. Glycoproteins of similar molecular weight have been reported in a number of other studies of cell-matrix interactions (Oppenheimer-Marks & Grinnell, 1984; Docherty et al., 1981; Kurkinen et al., 1984).

Based upon the calcium-dependent stability to trypsin, Oppenheimer-Marks and Grinnell (1984) have proposed that a 48 Kd wheat germ agglutinin-binding glycoprotein acts as potential fibronectin receptor. It should be noted that a 48 Kd glycoprotein was also reported to be retained by cytoskeletons of BHK fibroblasts and embryonic chick cells after extraction with non-ionic detergent (Docherty, Dysart & Edwards, 1981). The relation between these 47 Kd and 48 Kd glycoproteins and the 47 Kd gelatin-binding protein "colligin" (Kurkinen et al., 1984) is currently under investigation.

#### Inhibition by gangliosides

Kleinman et al. (1979) showed that gangliosides from bovine brain prevented the attachment of trypsinized hamster ovary cells to urea-denatured collagen. Inhibition of attachment could be obtained with fibronectin pre-incubated with gangliosides and washed, suggesting that the gangliosides act by binding to fibronectin. The authors suggested that sialic acid-containing glycoconjugates (gangliosides or glycoproteins with similar oligosaccharides) at the cell surface may act as the



fibronectin receptor.

Other evidence favouring a role for gangliosides in the interaction of cells with fibronectin is the finding that their addition to ganglioside-deficient cells in serum-free culture causes appearance of fibrillar arrays of fibronectin, just as when cells adhere to a lattice of fibronectin adsorbed to an artificial substratum (Fishman et al., 1981).

Yamada et al. (1981) examined the hypothesis that glycolipids might serve as receptors for fibronectin, using three different biological assays systems (fibronectin-mediated spreading of BHK cells, fibronectin-mediated effects on transformed cells using the SV1 tumour cells, and haemagglutination). They demonstrated that certain gangliosides inhibit fibronectin-mediated haemagglutination, cell spreading, and restoration of a normal morphologic phenotype to transformed cells. These findings support the notion that the receptors for fibronectin on the cell surface either consist of or contain gangliosides or other negatively charged lipids. However, Rauvala et al. (1981) found that similar concentrations of the ganglioside GT1b (0.25 - 0.5 mM) inhibited the attachment of Nil cells to adsorbed fibronectin, but that GT1b at slightly higher concentrations also inhibited adhesion to adsorbed soy-bean lectin.

To examine surface components which may mediate binding, Schwarz et al.(1984) treated Fn beads with purified glycosaminoglycans (GAGs) or glycolipids prior to incubation with cells. Gangliosides GT1, and GM1 inhibited bead binding induced by the presence of anti-Fn antibody, by certain types of glycosaminoglycans, and by sialylated glycolipids. From these findings, the authors suggested that although glycolipids block bead binding they are not the endogenous binding site for FN, and protease sensitive components (glycoproteins or proteoglycans) may be more likely candidates as cell surface-binding sites for fibronectin.

#### Use of antisera or monoclonal antibodies

Another approach which might in principle lead to the identification of the fibronectin receptor is the use of antibodies which block cell-substratum adhesion. Tarone et al.(1980) and Hughes & Pena (1981) reported that antibodies directed against cell-surface glycoproteins of BHK-21 cells can inhibit attachment and spreading of the cells on fibronectin coated substratum. The major component with which the antibodies reacted specifically was a trypsin-resistant 130 Kd-140 Kd glycoprotein. Further support for the notion that the receptor is a protein comes from studies showing that treatment of cells with certain proteases, pronase, proteinase K, chymotrypsin, papain, subtilopectidase A, and thermolysin, abolishes the ability of cells to attach to fibronectin ( Tarone et al.,1982).

A glycoprotein with molecular weight 130 Kd and 140 Kd has also been identified in BHK-21 cells by Wylie et al.(1979) using antisera to induce cell rounding and detachment from solid substrata. It is possible that Tarone et al.(1980) and Hughes et al. (1981) are discussing the same glycoprotein described by Wylie et al.(1979), because they have the same molecular weight. Oppenheimer-Marks et al. (1982) reported that antibodies, and Fab fragments of antibodies, produced against WGA-binding proteins of BHK cells reacted primarily with polypeptides of molecular weights 48 Kd, 61 Kd, 63 Kd, 83 Kd, 105 Kd, 120 Kd, 165 Kd, 210 Kd, and 230 Kd. These antibodies were considered to interfere with BHK cell fibronectin receptors on the basis of the ability of the IgG or Fab fragments to inhibit cell spreading on Fn-coated substrata, cause rounding and detachment of cells previously spread, and also inhibit binding of Fn-coated latex beads to the cells.

By using antisera or monoclonal antibodies, a group of membrane glycoproteins with molecular weight ranging from 120 Kd to 160 Kd have been identified as being involved in adhesion of mammalian, as well as avian fibroblasts (Giancotti et al.,1985).

### The use of affinity chromatography

This approach depended on the discovery of inhibition of cell attachment by fragments of fibronectin.

Yamada et al.(1984) found that fibronectin-mediated adhesion as measured by a standard cell-spreading assay can

be inhibited by fibronectin itself at very high concentration, for example 10 mg/ml, similar inhibition is produced by a purified cell-binding fragment of 75 Kd and two synthetic peptides from this region, but not by a series of other proteins and peptides. The inhibition appears to be competitive, and can be overcome at least partially by higher concentrations of adsorbed fibronectin or by longer incubation times, and similar inhibition is found in an assay measuring fibronectin-mediated cell attachment to type I collagen, but not by lectin.

Pierschbacher et al. (1984) have isolated a peptic fragment of molecular weight 11.5 Kd. This domain contains all the cell attachment activity to be found among the proteolytic fragments of the fibronectin molecule.

By using synthetic peptides modelled from the sequence of this domain, Pierschbacher et al. (1983) described a 30 amino acid synthetic peptide which carries the cell attachment-promoting activity.

They localized the function of the the fibronectin to a sequence of only four of these amino acids L-arginyl-glycyl-L-aspartyl-L-serine. The same tetrapeptide, L-arginyl-glycyl-L-aspartyl-L-serine is present in some other proteins which may interact with cells, including fibrinogen (Watt et al., 1979), a surface protein of Escherichia coli (Clement et al., 1981), and a Sindbis virus coat protein (Rice et al., 1981).

Pierschbacher et al. (1984) reported that the Arg-Gly-Asp sequence must be maintained to preserve activity, while some variation at the position occupied by the serine residue is compatible with activity.

Knowledge of these sequences allowed these workers to identify a convincing candidate for the role of a fibronectin receptor: the 140 Kd glycoprotein obtained by Pytela et al. (1985) by affinity chromatography.

Thus in order to identify the receptor, an affinity column was prepared by coupling to CNBr-activated Sepharose a 120 Kd chymotryptic fragment of fibronectin that does not bind to gelatin or heparin, but retains cell-attachment promoting activity. An octylglucoside extract of cells was applied to the affinity matrix. The column was then eluted with the synthetic peptide Glycyl-L-Arginyl-Glycyl-L-Aspartyl-L-Proline, which contains the cell attachment recognition sequence of fibronectin. A 140 Kd protein was specifically eluted, which appears to be an integral membrane protein, closely associated with matrix fibronectin. Thus, it has been suggested that this protein is a cell surface receptor involved in adhesion of cells to fibronectin.

#### 4. Laminin

Laminin, a major glycoprotein of basement membranes, has been investigated in relation to a wide variety of

biological phenomena such as cell attachment, cell growth and differentiation, morphogenesis and cell migration, and cancer metastases.

Laminin was isolated by Timpl et al. (1979) by extracting the extracellular matrix of chondrosarcoma EH mouse strain with 0.5 M sodium chloride at neutral pH. Laminin was separated from fibronectin on DEAE-cellulose, and antibodies against the two glycoproteins did not cross-react. By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reduced and denatured, laminin migrates as two major components of 200 Kd ( $\alpha$  subunit) and 400 Kd ( $\beta$  subunit).

The  $\alpha$ -subunit was isolated and its structure was studied (Rao et al., 1982). Using  $\alpha$ thrombin selectively, the  $\beta$  subunit of laminin was removed without altering the size or amount of the  $\alpha$  subunit. The authors found that the  $\alpha$  subunit mediated the attachment of human carcinoma cells to type IV collagen.

Terranova et al. (1980), investigating the adhesion of PAM 212 transformed mouse epithelial cells to different types of collagen, found that laminin strongly promoted attachment to collagen type IV, but not to types I, II, III, or V. Fibronectin was inactive with these cells. They reported that the receptor for laminin on the surface of these tumour cells may be involved in the initial interaction of tumour cells via laminin with the vascular

basement membrane to facilitate invasion and subsequent promotion of metastasis.

Johansson et al.(1981) found that both laminin and fibronectin adsorbed to culture substrates would support the attachment and spreading of adult rat hepatocytes. Carlsson et al.(1981) found that cells from normal and regenerating mouse liver responded equally to fibronectin, cells from regenerating liver were more adhesive than normal cells to laminin, a change reversed when regeneration ceased.

Protease-derived fragments (pepsin and cathepsin G) of laminin have been utilized to identify the domains of the laminin molecule that participate in the attachment of human breast carcinoma cells to type IV collagen (Terranova et al.,1983). Protease fragments were isolated by high-performance liquid chromatography and studied by gel electrophoresis. The authors reported that the major cell-binding domain was found to reside near the intersection point of the short arms, where the type IV collagen-binding domain was associated with the globular end regions of the short arms.

Laminin receptor isolated from human breast carcinoma cells or from mouse melanoma cells has a Kd of 2 nM and a molecular mass approx. 67000D with 50,000 - 100,000 receptors per cell, and was used as an antigen to generate monoclonal antibodies( Liotta et al.,1985).

By using affinity chromatography, a glycoprotein of molecular weight 69 Kd was isolated from murine fibrosarcoma cells (Malinoff et al., 1983). Laminin affinity chromatography was performed on a column of laminin coupled to Sepharose 4B. The column was then eluted consecutively with urea or glycine-HCl. The authors reported that the 69 Kd glycoprotein is a subunit or component of a larger cell surface receptor protein for laminin and may mediate the interaction of the cell with its extracellular matrix.

Moreover, Lesot et al. (1983) isolated a glycoprotein with an apparent molecular weight 68 Kd by affinity chromatography of muscle cell plasma membranes from mouse thigh and rat L6 myoblasts on laminin Sepharose. They also identified laminin-binding components in the muscle cell surface. Proteins of plasma membranes from mouse muscle and from rat L6 myoblasts were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose paper by electroblotting. The paper was incubated with <sup>125</sup>I labelled laminin, and a prominent band of approximate molecular weight 68 Kd was revealed. They proposed that 68 Kd glycoprotein is involved in the interaction of myoblasts with laminin substrates and may serve as anchorage site for laminin on the surface of skeletal muscle cells.

## 5. Chondronectin

Cartilage does not contain fibronectin (Dessau et al., 1978; Stenman & Vaheri, 1978), and purified fibronectin does not promote the adherence of freshly isolated



does not promote the adherence of freshly isolated chondrocytes to tissue culture dishes, whereas fibronectin-free serum does (Hewitt et al., 1980). One group has described the isolation of a chondrocyte-attachment factor in serum, "chondronectin", which can be separated from fibronectin by DEAE-cellulose chromatography (Hewitt et al., 1981). The chondronectin isolated from serum has a molecular weight of 180 Kd and contains disulphide-linked chains, and was suggested to be analogous in function to fibronectin, but specific for chondrocytes. For example neuritic regeneration from cultured peripheral and central neurons is promoted by laminin (not by fibronectin) (Manthorpe et al., 1983).

#### 6. Serum spreading factor (vitronectin)

A very large number of observations suggested that fibronectin is not the only adhesive proteins in plasma or serum (Hewitt et al., 1980; Barnes et al., 1980; Pytela et al., 1985). Human plasma and serum were found to contain another distinct protein, referred to as "serum spreading factor" with adhesive properties similar to those of fibronectin (Holmes, 1967).

Serum spreading factor is a glycoprotein component of human serum that promotes the attachment and spreading of a wide variety of both fibroblastic and epithelial cells in vitro (Barnes & Sato, 1979; Barnes et al., 1980, 1981, 1982 a).

procedures:

1. Adsorption to glass bead column (Barnes et al., 1981). Serum spreading factor of molecular weight 70 Kd (named vitronectin) was isolated.
2. Chromatography on insolubilized monoclonal antibodies and heparin (Hayman et al., 1983). Two molecular species of apparent mass 65 Kd and 75 Kd were isolated.
3. Adsorption to glass beads followed by chromatography on Con A-Sepharose, DEAE-agarose, and heparin-agarose (Barnes et al., 1983). Spreading factor with molecular weights 65 Kd and 75 Kd was prepared from human fetal membranes.

The isolated serum spreading factor 'vitronectin' (Barnes et al., 1981, 1983; Hayman et al., 1983) has been shown to exhibit cell attachment and spreading promoting activity and to bind monoclonal antibody to human plasma.

A vitronectin receptor was isolated (Pytela et al., 1985b) by using affinity chromatography. Detergent extracts of human osteosarcoma (MG-63) cells were applied to either vitronectin-Sepharose or Sepharose linked to the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro, which includes the fibronectin cell attachment sequence Arg-Gly-Asp. Two molecular species of apparent 125 Kd and 115 Kd were eluted with a solution containing the Gly-Arg-Gly-Asp-Ser-Pro peptide.

## 7. Collagen receptor

Collagen is the major extracellular matrix component contributing to the visco-elastic properties of tissues, and has been reviewed in detail by Ramachandran (1967), Ramachandran & Raddi (1976), Viidik & Vuust (1980), and Kleinman, Klebe & Martin (1981).

Collagen is a trimeric glycoprotein of approximately 100 Kd subunit molecular weight. It has been isolated in five types (type I-V) from various tissues, each type is distinguished on a number of criteria including the amino acid sequence of the subunit, the amount of hydroxyproline and hydroxy-lysine, and the degree of glycosylation.

The existence of collagen receptors on the cell surface was first suggested from binding studies of radiolabelled collagen molecules to membranes of non-connective tissue cell types. Koehler et al. (1980) described a collagen-binding membrane glycoprotein of molecular weight 40 Kd in the acrosomal region of rabbit spermatozoa, while Chiang et al. (1982) reported the isolation of a collagen-binding membrane protein of molecular weight 65 Kd from platelets.

A collagen-binding glycoprotein was isolated from chick chondrocyte surface membranes by affinity chromatography on type II collagen-Sepharose (Mollenhauer et al., 1983). Solubilized plasma membrane from sternal cartilage of chicken was applied to pepsin extracted native

type II collagen coupled to a Sepharose column. The column was then eluted with a linear gradient of NaCl. The eluted fractions were dialyzed against sodium deoxycholate (DOC) and rechromatographed on type II collagen Sepharose. The fractions were analyzed by SDS-PAGE gel.

The purified glycoprotein has an apparent molecular weight of 31 Kd and binds to native chick collagen type I,II,III, and V, and it is referred as anchorin CII (anchoring of chondrocytes to type II collagen).

The collagen-binding component of chondrocytes, anchorin CII, has been found to contain a fucose rich containing glycoprotein and exhibits the properties of an integral membrane protein. This concept is supported by the observation of capping and patching of antibodies on the surface of matrix-free chondrocytes, which is induced by antibodies at elevated temperatures (Mollenhauer et al.,1984). The authors proposed that the anchorin CII serves as anchorage site for extracellular collagen to the chondrocyte membrane and may be involved in cell-matrix interactions in cartilage.

### III. METHODS USED TO MEASURE CELL-SUBSTRATUM ADHESION

Various attempts have been made to measure cell substratum adhesion. Most cell-substratum adhesion assays are variations on the general principles of applying a distractive force to cells that have been permitted to form adhesive contacts, and then either the proportion of cells that resist distraction, or the force required to remove all the cells is measured. It is difficult to separate the force required for cell deformation (Weiss, 1961) and drainage (Curtis, 1962).

Distractive forces such as a stream of fluid (Weiss, 1961), or a blast of air (Gail & Boone, 1972), and many others have been used.

The flow-chamber adhesion assay (Forrester & Lackie, 1984) differs from such assays. It measures attachment of cells to the chamber as a cell suspension is passed through and thus minimizes the contribution of spreading to adhesion. Using this assay, adherent cells can resist the fluid shear and spreading behaviour can subsequently also be monitored.

#### IV. SELECTION OF VARIANTS AS A MEANS TO INVESTIGATE THE MECHANISM OF CELL-SUBSTRATUM ADHESION

Variant cell lines selected for poor adhesion to various substrata should be very important in analysis of the role of surface receptors, and appropriate cytoskeletal response in adhesion of cells to substrates. A problem with this approach is that selection for poorly adherent cells imposes selection against anchorage-dependence.

Two types of selection have yielded mammalian cell variants with altered adhesiveness to substrates. First, there are cell variants selected directly for an altered ability to adhere to serum- (Klebe et al., 1977), or fibronectin-coated substrata (Harper & Juliano, 1980; Oppenheimer-Marks et al., 1984). Second, some variant cells selected for lectin resistance show low adhesion to substrates (for detail see Stanley, 1980).

##### A. Selection of mutant cells defective in adhesion

A number of workers have selected and described mammalian cell variants with altered adhesiveness for substrates (described in detail below).

A cell attachment mutant of the Chinese hamster ovary cell line (CHO) was isolated by enriching a wild-type CHO population with non-attaching cells by collecting cells that did not adhere to collagen (Klebe et al., 1977). In

brief, cell attachment protein (cCAP) (now known to be fibronectin) was permitted to bind to collagen-coated plates. Dulbecco's medium containing 10% foetal calf serum was then added to the collagen coated plates and was pre-incubated. Following the pre-incubation, CHO cells were added to the treated plates. The non-attached cells were removed and cultivated. The above procedure was repeated several times over a period of two months at which time a mutant population became clear. The mutant cells were cloned in semi-solid agar in order to obtain a pure population of the attached mutant.

The mutant isolated, CHO att-, was found to be defective in its attachment to serum or fibronectin bound to denatured collagen films. However, this variant could attach if much larger amounts of fibronectin (1000  $\mu\text{g/ml}$ ) or 100% serum were used or if the divalent cation concentrations were increased, for example from 1 mM to 10 mM Mg and from 1 mM to 100 mM Ca .

Pouyssegur et al. (1977) have isolated two mutants of Balb/c 3T3 cells (Ad6 and Ad8) defective in adhesion to tissue culture dishes in the presence of serum. Mutagenized cells were pre-incubated with prostaglandin E1 and methyl-3-isobutylxanthine, a treatment which raises intracellular cyclic AMP and maximises the adhesion of wild-type cells.

Poorly adherent cells were then enriched by detachment with a minimal exposure to trypsin. The final

selection was made by taking only those cells which were detached by gently shaking in the absence of trypsin. The two clones AD6 and Ad8 were found to be more rounded (less well spread) than wild type in sparse culture.

The AD6 cell line is characterized by low adhesion to substratum, round shape, increase in surface microvilli, increase in agglutinability by concanavalin A, and loss of directional motility. These properties are often observed in transformed cells. However, AD6 has normal growth properties, including anchorage-dependence, does not form tumours, and is considered not to be transformed.

Subsequent studies of AD6 identified a specific metabolic lesion (Pouyssegur & Pastan., 1977). The mutant cells were found to incorporate exogenous D-glucosamine into macromolecules much more slowly than wild type, and to accumulate non-acetylated glucosamine 6-phosphate, rather than UDP-N-acetylhexosamines. The enzyme responsible for the acetylation step, glucosamine phosphate acetyltransferase, was assayed in wild type cells but was undetectable in AD6. Adhesion and spreading are restored to the levels of the wild type by supplying the mutant with exogenous N-acetylglucosamine.

The cell-to-substratum contacts of AD6 have been examined by Yates & Izzard (1981), using interference reflection microscopy. Both AD6 and Balb/c 3T3 formed close contacts, in which the cell surface is separated from the



substrate by about 30 nm, but focal contacts (10-15 nm separation) although present in wild type were almost completely absent in the mutant.

Harper and Juliano (1980) have described a CHO variant (ADv) selected for reduced adhesion to serum-coated tissue culture plates. Cells were grown in suspension and then were exposed to the mutagen, ethane methane-sulphonate, (EMS). The mutagenized cells were then plated in serum containing medium to enrich for less adhesive phenotype.

The non-adhesive cells were recovered, and replaced in suspension culture and were grown back to the original cell density. The cells were then cloned and individual clones were tested for their ability to adhere. These clones displayed reduced adhesion to substrata composed of collagen layers coated with bovine serum or with fibronectin.

Harper and Juliano explained their results by suggesting that this variant (ADv) clone may have a cell surface defect that prevents it from utilizing exogenous fibronectin as an adhesion-promoting ligand. However the ADv cells seem to have normal cytoskeletal and metabolic capacity that allow them to attach and spread on substrata coated with Con A or poly-L-lysine. In addition, capping of FITC-Con A is similar in wild type and ADv cells (Harper & Juliano, 1980).

Briles and Haskew (1982) isolated variants of rat hepatoma cell (HTC) selectively defective in their ability to attach to collagen. The selection was carried out as follows: Rat hepatoma cells were seeded on collagen coated dishes and incubated. The supernatant fluid containing a subpopulation of non-adherent cells was transferred to culture flask supplemented with growth medium.

After successive rounds of selection on collagen in HBSS, clones were isolated and examined for their ability to attach to collagen. A variant was isolated which exhibited normal attachment and spreading behaviour on fibronectin substrata, but had decreased ability to attach to native collagen.

Oppenheimer-Marks et al. (1984) have isolated and described an adhesion defective mutant of BHK cells (FN-1). Baby hamster kidney cells (a sub-line adapted to grow in suspension) were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine for one day and were subjected to selection by an enrichment procedure to obtain a population of non-attached cells.

Poorly attached cells were collected and resuspended in medium containing calf serum and were incubated for 2 hours at 37°C. The non-attached cells were then grown in suspension culture. A selection procedure was carried out 15 times until 95% of the cell population was unable to attach to tissue culture flasks in MEM containing 10%

serum. The cell variant FN-1 was then cloned from non-attached cell population, recloned and tested in different assays of fibronectin receptor function such as cell attachment, cell-substratum spreading, binding and phagocytosis of fibronectin coated beads.

The variant cell, FN-1, was shown to have decreased cell attachment compared to parental cells and were unable to spread, but were able to attach and spread on substrata coated with polycationic ferritin, Con A or anti-BHK cell surface antibody. The authors suggested that variant FN-1 cells have a defective fibronectin receptor. Now that techniques are available for detection of fibronectin receptors (Pytela et al., 1985) this could be investigated.

Takasaki et al. (1984) selected variants of a murine macrophage-like cell line J 774 presumed to be deficient in general phagocytosis, by selecting cells not killed by uptake of daunomycin derivatized beads.

In view of similarities between phagocytosis and deficiency spreading it might be expected that variants selected for phagocytosis would have low adhesion for substrates. However, the authors reported that variant cells showed normal adhesiveness and suggested that there is a difference between adhesiveness to plastic and ingestion of beads, as there is between binding to Fc receptor and Fc mediated ingestion.

## B. Lectin-resistant variants

Many cell lines have been selected for an ability to grow in the presence of toxic plant lectins. It is assumed that lectins interact with carbohydrate moieties of glycosylated membrane macromolecules. Lectins are cytotoxic to many cultured cells, which therefore suggests their use as selective agents in obtaining membrane mutants of mammalian cells with reduced or altered glycoprotein on the surface.

The mechanism of toxicity of lectins to cells (with exception of ricin) appears not to be understood, but resistance is acquired by alterations in surface glycosylation (reviewed by Stanley, 1980). Ricin and phytohemagglutinin (PHA)-resistant mutants are poorly spread and weakly adhesive to culture substrates, whilst WGA-resistant mutants (for example WGA-resistant CHO cells) are well spread and adhesive to culture substrates .

Meager et al.(1976) isolated a series of ricin-resistant variants of BHK cells in a single step selection after mutagenesis, many of which showed the phenotypic character of weak adhesion to the culture surface. Edwards et al.(1976) examined in some detail the adhesive properties, both cell-to-cell and cell-to-substrate adhesion of 8 of these lines. It was found that 6/8 were strikingly less adhesive to substrates than parental wild type cells.

Mutants isolated by resistance to PHA are not cross-resistant to ricin, and vice-versa. PHA-resistant CHO cells, which are very poorly spread in culture are highly resistant to wheat germ agglutinin, but more sensitive than wild type to ricin.

Some variants appear to be deficient in a specific N-acetylglucosaminyl transferase activity. In CHO cells, this was shown to be one of the transferases specific for transferring N-acetylglucosamine residues to the core of asparagine-linked oligosaccharides of glycoproteins (Stanley, 1980).

The isolation of poorly metastatic tumour cell mutants from metastatic wild type cells by selection in vitro for resistance to the toxic effect of lectins such as wheat germ agglutinin supported the notion that certain surface oligosaccharides are necessary for expression of the metastatic phenotype and these mutants showed greater attachment ability to fibronectin and type IV collagen-coated surfaces than metastatic parentals (Dennis et al., 1984).

Finne et al. (1982) described a wheat germ agglutinin (WGA)-resistant mouse melanoma cell line with many altered properties, including increased fucose content and decreased sialic-acid-content. This was explained by an increase in a specific fucosyltransferase activity.

WGA-resistant (WGA ) mutants of the PG19 melanoma (Bramwell et al.,1978), the B16 melanoma (Tao & Burger,1977) and the MDAY-D2 tumour line (Dennis et al.,1984) have shown to be less malignant than the parental tumour cell line, but mutants selected with other lectins such as ricin, PHA, Con A have shown either no reductions in their metastatic potential or even an increase (Reading et al.,1980; Tao et al.,1982). The mutant AD6 appears to be unique instance of a cell selected for low adhesion to the culture substrate, which was then found to have a glycosylation defect.

Since the attachment and spreading of the respective wild type cells is promoted by fibronectin, one interpretation of the properties of the various glycosylation-defective lines is that the N-linked oligosaccharides are required for the cells to bind to fibronectin (Pena & Hughes, 1978).

### Role of glycosylation

The effect of neuraminidase on intercellular adhesion and the low intercellular adhesiveness of ricin-resistant BHK 21 cells can be brought together by supposing that the form of glycosylation required for cells to be adhesive is complex-type asparagine-linked oligosaccharide in which one or more of the tri-antennary galactose residues is not sialylated.

The underglycosylation in variant cells, in some cases involving the peripheral "antennae" of complex-type N-linked glycosylation, does cause major reductions in cell-substrate adhesion of these cells and probably affects intercellular adhesion also. Since adhesion to substrates of these mutant cells can often be reverted by exogenous fibronectin, either binding of cells to fibronectin is not affected, or it is shifted towards weaker binding.

The following different mechanisms have to be considered:

1. The N-linked glycosylations on many different or certain specific glycoproteins are directly recognized and bound by substrate-attachment molecules such as fibronectin.

2. The oligosaccharides are required for the delivery of cell-cell adhesion molecules and matrix receptors to the cell surface or their stabilization from cleavage by proteases.

3. Oligosaccharides influence physical properties of, or interactions between, individual glycoprotein molecules which control clustering at points of adhesion.

Binding mechanisms 2 and 3 could involve endogenous lectins.

## AIMS OF RESEARCH

At the start of this work it was not clear whether interactions of plasma membranes with molecules such as the fibronectin and collagen are mediated by specific receptors in the plasma membrane and what the identity of these receptors might be. It was proposed that fibronectin molecule first recognises a specific sequence of amino acid on the collagen and another region recognizes the cell surface. To investigate these problems, I set out to isolate variants of anchorage-independent cells with low adhesiveness either to adsorbed serum films or to specific substrata such as fibronectin. Variants demonstrate examples of altered cytoplasmic and cell surface behaviour. These will likely prove to be useful in determining the identity of the cell surface receptor and the appropriate cytoskeletal response. Subsequently putative receptors of fibronectin, laminin, vitronectin and collagen II have been identified by others (discussed above).



Chapter Two

MATERIALS AND METHODS

## Materials

### Cell line

Polyoma virus transformed BHK-21 cells (Py3 BHK), derived from PyY (Stoker & McPherson, 1964), recloned in soft agar as previously described (Edwards et al., 1979).

### Media

The media most frequently used throughout this work are specified below.

### Hepes saline

For 5L

NaCl	40 g
KCl	2 g
D-glucose	5 g
Phenol red (1/2%)	10 ml
Hepes	11.92 g

pH adjusted to 7.2 with N NaOH

Hepes is N-2hydroxethyl piperazine-N'-2-ethane sulphonic acid (Cambridge Research Biochemicals).

### Hanks Hepes

For 5L

NaCl	40 g
KCl	2 g
D-glucose	5 g

Phenol red (1/2%)	10 ml
Hepes	11.92 g
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.93 g
MgCl <sub>2</sub> .6H <sub>2</sub> O	1.0 g
pH adjusted to 7.2	

#### Disaggregation agents

1. Versene: 0.55 mM EDTA (ethylene diamine tetra acedic acid) in phosphate-buffered saline, pH adjusted to 7.4.
2. Trypsin: 0.25% W/V (Difco 1:250) in Tris-saline. pH 7.4.

#### Tryptose phosphate broth (TPB)

147.5 g TPB (Difco) dissolved in 5 L double-deionised water and pH adjusted to 7.4.

#### Bicarbonate Buffered Eagles Medium (ECT)

Sterile water	135 ml
X10 concentrate Glasgow modified Eagles medium (Flow Laboratories).	16 ml
GP5A [glutamine (200mM), Penicillin (200,000units/ml), streptomycin (200,000 units/ml), Amphotericin B: Flow laboratory].	5 ml
7.5 % NaHCO <sub>3</sub>	5 ml
Calf serum	20 ml
TPB	20 ml

Cells grown in ECT were equilibrated with a gas phase of 95% air, 5% CO<sub>2</sub> mixture for buffering purposes.

Ham's F10

180 ml	20mM Hepes water
20 ml	X10 concentrate Ham's F 10 (Flow)
1 ml	7.5% NaHCO <sub>3</sub>
5 ml	GPSA
20 ml	Foetal calf serum
20 ml	TPB

Phosphate Buffered Saline (PBS a)

For 1 L

NaCl	9.86 gm
KCl	0.25 gm
Na <sub>2</sub> HPO <sub>4</sub>	1.44 gm
KH <sub>2</sub> PO <sub>4</sub>	0.25 gm
pH adjusted to 7.2	

Buffered Formalin

For 1 L

Formaldehyde (40%)	100 ml
NaCl	8.0 gm
KCl	0.2 gm
Na <sub>2</sub> HPO <sub>4</sub>	1.15 gm

$\text{K}_2\text{HPO}_4$

0.2 gm

pH adjusted to 7.2

### Culture Dishes

30 mm diameter culture dishes were obtained from Sterilin Ltd, 25 cm<sup>2</sup> culture bottles from Falcon plastics Ltd and glass roller bottles (800 cm<sup>2</sup>)

### Blotting Buffer

Tris 25 mM

Glycine 192 mM

Methanol (20%) 800 ml

pH adjusted to 8.3 and made up to 4 L with distilled water.

### Materials for Microsomal Fractions

TMS in 1 L

Tris 5.0 mM

Sucrose 0.25 M

$\text{MgSO}_4$  0.2 mM

pH adjusted to 7.4

Phenyl methyl sulphonyl fluoride (PMSF) 0.2 mM,

Trasyolol 25 units/ml.

## Materials for Cytoskeleton Preparation

1. Hanks HEPES (HH)
2. Hanks HEPES containing the following protease inhibitors:  
2 mM phenyl methyl sulphonyl fluoride (PMSF) (Sigma).  
1 mg/ml p-Tosyl-l-arginine methyl ester-HCl (TAME) (Sigma).
3. Hanks HEPES containing the following:  
1% Triton X-100 [polyethylene glycol (9-10) p-t-octyl phenol] (Sigma).  
2 mM PMSF  
1mg/ml TAME
4. Tris-saline containing the following:  
0.5% Triton X-100  
10 mM magnesium chloride  
0.1 mg/ml deoxyribonuclease I (Sigma). Specific activity 1500 kunitz units per mg protein.
5. "High Salt " solution  
Hanks HEPES containing 1.5 M potassium chloride.

## Polyacrylamide Gel Electrophoresis (PAGE)

### A. Reagents

All reagents were electrophoresis grade and were obtained from the following sources:

Acrylamide and bis acrylamide from Koch-light Lab.

Sodium dodecyl sulphate (SDS) and Coomassie blue from BDH.

Ammonium persulphate, bromophenol blue, and N,N,N',N',-tetra methylethylene diamine (TEMED) from Bio-Rad.

Tris (hydroxymethyl) aminomethane (Tris) and glycine from Sigma.

## B. Stock solutions

### 30% acrylamide:

acrylamide	28.5 g
bis acrylamide	1.5 g
distilled water	100 ml

### Running gel buffer:

Tris	18.15 g
SDS	0.4 g

dissolved in 50 ml distilled water, pH adjusted to 8.9 with HCl, and made up to final volume of 100 ml with distilled water.

### TEMED:

N,N,N',N',Tetra methyl ethylene diamine.

### Ammonium persulphate:

10% W/V in distilled water freshly made up before use.

### Stacking gel buffer:

Tris	5.9 g
SDS	0.4 g

dissolved in 50 ml distilled water, pH adjusted to 6.7 with HCl, and made up to 100 ml with distilled water.

Upper tank buffer:

Prepared as a 5x concentrate, diluted for use in distilled water.

Tris 31.6 g

Glycine 20.0 g

SDS 5.0 g

in one litre of distilled water.

Lower tank buffer:

Prepared as a 5x concentrate, diluted for use in distilled water.

Tris 60.5 g

SDS 5.0 g

pH adjusted to 8.1 with HCl and made up to a final volume of one litre with distilled water.

Protein solubilizing medium (boiling mixture):

stacking gel buffer 2.5 ml

$\beta$ -mercaptoethanol (Koch-Light) 2.5 ml

SDS 1.0 g

glycerol (BDH Ltd) 2.0 ml

Bromophenol blue 10.0 mg

Made up to 10 ml with distilled water.



After addition of the boiling mixture, samples were placed in a boiling water bath for 3 minutes, used or stored at -20°C. Used in a ratio of 4 parts sample to 1 part boiling mixture.

Protein staining solution:

Coomassie brilliant blue stain (BHD Ltd) was made up to a 0.1% W/V solution by dissolving in methanol: acetic acid: water, ratio 50: 7: 50.

Destaining solution:

This was a methanol: acetic acid: water  
50 : 70 : 880

Molecular weight standards:

These following proteins were used at concentration of 1 mg/ml.

Fibronectin (bovine serum)	220 K
β-galactosidase	120 K
Phosphorylase a	95 K
Catalase	60 k
Oval bumin	45 K
Concanavalin A	22 K

### Buffer A

For rinsing <sup>125</sup>I-Con A labelled gel (Burridge, 1976)  
was

Tris	50 mM
CaCl <sub>2</sub>	1 mM
MagCl <sub>2</sub>	1 mM
pH adjusted to 7.4 with HCl	

### Fibronectin

For coating coverslips and culture bottle surfaces, fibronectin was isolated from bovine serum on a gelatin-Sepharose column using the method of Engvall & Ruoslahti (1977) and was checked by SDS-PAGE.

### Concanavalin A (Con A) and FITC Con A

were obtained from Sigma. [<sup>3</sup>H] Thymidine, with specific activity 5 Ci/m mol (185 GBq/ m mol.), used for labelling cells was obtained from Amersham International Ltd-Bucks.

Iodine (carrier free) used for the iodination of Con A and Fn [usually 1 mCi (37 MBq)] was obtained from Amersham International Ltd-Bucks.

### Actin stain

NBD-Phalloidin (Molecular Probes Inc. Plano Texas, USA).

Scintillation fluid

Aquasol was obtained from New England Nuclear and dispensed in 10 ml aliquots.

## METHODS

## Cell culture

Cells were grown attached in culture bottles in modified Eagles minimal essential medium supplemented with 10% calf serum and 10% tryptose phosphate broth (ECT) at 37 °C with 5% CO<sub>2</sub>, 95% air as gas phase.

Cells were subcultured when they reached confluency by pouring off the ECT and washing the monolayer twice with HS, cells were then trypsinized off. The tryptic activity was stopped by the addition of 5 mls fresh ECT, and the cells were shaken off into this medium, were counted in a haemocytometer and replated at the required density. Cells were discarded after 6 weeks of serial propagation.

## Selection

The transformed cells used for selection were Py-transformed BHK 21 cells which had undergone about 12 passages in (non-selective, attached) culture since recloning in soft agar.

To mutagenise the cells, MNNG (Meager et al., 1975) at 0.5 µg/ml was added to 2/3 confluent cultures in ECT for one day. Mutagenized cells were grown for a further 2-3 days to optimum density (several generations). 30 million mutagenized Py3 cells were grown overnight in roller culture bottles (800 cm<sup>2</sup>) in ECT. On the second day, the cells which were not attached during roller culture or

those could be released by a gentle rinse with growth medium (about 10 million) were recovered by centrifugation and then were distributed between 2 plastic culture flasks 75 cm<sup>2</sup>) previously coated with bovine plasma fibronectin (5 mls, 25 µg/ml in Hepes-buffered Hanks medium, for 30 minutes).

After 5-6 hours at 37°C, the unattached cells were transferred in 30 mls ECT to another (single) 75 cm<sup>2</sup> flask (not precoated) and incubated overnight. The procedure described for the second day was repeated on days 3 and 4. After the 6 hour incubation on day 4, the unattached cells were plated on dishes for colony counts and morphology inspection.

#### Growth of cells in soft agar

An agar underlay was prepared in 90 mm bacterial grade plastic petri dishes with Hams medium containing 10% foetal calf serum, 10% TPB and 0.5% Noble agar. This was overlaid with 1.5 mls of Hams containing 0.3% Noble agar and cells in a range of concentrations. The dishes were incubated at 37°C with 5% CO<sub>2</sub>, 95% air as the gas phase until colonies developed.

#### Determination of chromosome number

Py3, mutants and mouse capillary endothelium cells were grown near confluent in ECT. 10 µg/ml colcimide in HH

was added to the growing culture, and incubated for 4 -5 hours, to cause cells to accumulate in mitosis.

Cells were trypsinized and resuspended in graduated conical-tipped centrifuge tubes containing ECT. Cells were recovered by centrifugation at 1000 rpm for 7 minutes, then resuspended in 10 mls preheated hypotonic medium (ECT 1: Distilled water 2). Cells were harvested by centrifugation, resuspended in 2 mls hypotonic medium for 4 - 5 minutes, and the volume was made up to 10 mls with Carnoys fixative (ethanol 3: acetic acid 1). Cells were mixed gently using parafilm to cover the tubes. Centrifugation was carried out for the third time and the cells were resuspended in 45% glacial acetic acid.

2-3 drops of cell suspension were released on chilled coverslips and were dried over the pilot flame of a bunsen burner by moving them to avoid cracking. Coverslips were stained for 10 minutes in Giemsa (1 : 10 in PBS), washed twice in PBS, air dried and then mounted on clean slides with Clear Mount. The chromosomes were examined and counted using x100 oil-immersion objective, and compared.

#### Fluorochrome staining for mycoplasma

Py3 and mutant cells were grown on coverslips in sector box containing ECT overnight in air/CO<sub>2</sub>.

Cells were fixed with acetic acid and methanol (1:3).

The coverslips were rinsed twice in distilled water. 2 mls bisbenzimidazole fluorochrome "Hoechst 33258" were added and incubated at 37°C for 30 minutes. The coverslips were rinsed thoroughly in distilled water, mounted on glass slide with PBS/glycerol 1:1 and examined using the fluorescence microscope.

#### Preparation of cells for spreading assay

Py3 and variant cells were grown in 25 cm<sup>2</sup> plastic tissue culture flasks until the cells formed a confluent monolayer. The medium of Py3 cells was poured off, the cell monolayer washed twice with HEPES saline, and 5 mls of trypsin/EDTA solution added for 45 seconds. This was poured off and the culture flask left for 5 minutes. Trypsin was then stopped with ECT. The variant cells were detached by a sharp tap (without use of trypsin or EDTA). Parental and variant cells were washed twice in Hanks HEPES by centrifugation at 1000 g for 7 minutes and resuspended in HH or HS at a concentration of  $5 \times 10^4$  cells/ml.

#### Preparation of coverslips for adhesion/spreading assay

Glass coverslips of 13 mm diameter were immersed in hot Decon 90 for 2 hours, rinsed in running tap water overnight, washed in distilled water, absolute ethanol, and sterilised.



Coverslips to be coated with fibronectin or other proteins were placed in sectorized boxes and 1 ml of protein solution at the desired concentration added and left for 1 hour at room temperature. The coverslips were then rinsed in Hanks Hepes and placed in clean sectorized boxes for use.

#### Py3 and variant cell spreading assay

1 ml of parental and variant cell suspension at  $5 \times 10^4$  cells/ml was added to each clean glass or coated coverslip. Cells were incubated for 150 minutes at  $37^\circ\text{C}$ , fixed in buffered formalin for 10 minutes, and stained with Coomassie blue for 10 minutes, rinsed in tap water, air dried, and mounted in Clear Mount. The degree of spreading was scored in five fields (using a X10 objective) on each coverslip and the minimum 200 cells per sample were scored. Cells were classified as being rounded, partially spread or fully spread.

#### Con A capping assay

Cells were grown on 13 mm coverslips overnight in growth medium. The medium was then poured off and cells were incubated with fluorescein isothiocyanate (FITC) conjugated Con A at  $37^\circ\text{C}$  for 60 minutes ( $50 \mu\text{g/ml}$ ). Cells were then fixed with 2% formaldehyde for 10 minutes at  $28^\circ\text{C}$  and were washed twice with PBS and mounted in PBS/glycerol 1:1 (vol/vol) on microscope slides.

### Scanning electron microscopy

Cells were grown on 13 mm coverslips overnight. The coverslips were washed with 0.2 M phosphate buffer, fixed with 2.5% glutaraldehyde solution and washed with buffer at 4 °C. Coverslips were then dehydrated with acetone and critical point dried from acetone with liquid CO<sub>2</sub>, in a Polaron 3000 critical point drier. Specimens were then coated with 200A gold using a Polaron SEM coating unit E 5000 and observed in a Phillips SEM 500.

### Flow-chamber adhesion assay

Flow-chamber assays of adhesion were performed as described by Forrester and Lackie (1984). Briefly, the flow-chamber was constructed between two microscope slides. The assembled chamber was placed on the stage of a Leitz inverted microscope maintained at 37°C by air curtain incubator.

The flow chamber surface was precoated by perfusion with serum (10%), fibronectin, or poly-lysine (25 µg/ ml in HH). Monodisperse suspensions of Py3 or variant cells in HH at  $0.5 \times 10^6$  cells/ml were perfused through the chamber at a flow rate of 0.02 mls/min (through a cross-section 0.15X4 mms). The velocity of the fluid stream was varied by doubling the speed of the plunger from 0.02 mls/min to 0.04 mls/min. Using a 10x objective, a phase-contrast image of the lower surface of the chamber is recorded on video-tape,

and the number of cells adhering can be counted on a sequence of frames, the moving cells are seen as a blur. The assay system permits measurement of both the early adhesion events and the late spreading behaviour of the cells.

#### Isolation of human serum spreading factor (vitronectin)

Serum spreading factor (vitronectin) was prepared from my own serum by adsorption on glass beads (size 75-150  $\mu$ , Sigma) as described by Barnes et al. (1981). The column was packed with acid washed glass beads and were equilibrated with 0.6M  $\text{NaHCO}_3$  (pH 8.0). 20 mls of serum was run on and the column was subsequently washed with two bed volumes of 0.6M sodium bicarbonate at pH 8.0 and one volume of distilled water.

The adsorbed serum spreading factor was eluted with a solution of 0.6M potassium bicarbonate and 0.2M potassium carbonate at pH 9.7. Fractions were collected and the protein peak detected by measuring absorbance at 280 nm. Fractions containing protein were pooled and adjusted to pH 7.4. Vitronectin was checked by SDS-PAGE, sterilized by filtration and frozen.

Upon thawing, some active precipitate was dissolved in sterile 10 mM KOH.

### Isolation of microsomal fraction

Microsome fractions were isolated as described by Gahmberg and Simons (1980). Confluent roller cultures (typically 6) were washed twice with Tris-buffered saline (Hepes), and the cells scraped from the glass into 5.0 mM Tris, 0.25 M sucrose, 0.2 mM  $\text{MgSO}_4$ , pH 7.4 (TSM). Cells were collected by centrifugation, washed once by resuspension in TSM and resuspended in 5X their pelleted volume of TSM containing 0.2 mM phenyl methyl sulphonyl fluoride and 25 units/ml /trasyolol.

The cell suspension was homogenized (Potter-Elvehjem), briefly sonicated and made 1.0 mM in EDTA. Nuclei and mitochondria were removed by centrifugation at 13,000 g for 15 minutes. A microsome fraction was sedimented (50,000 g, for 90 minutes), resuspended in 10 mls mM Tris, pH 8.6 and resedimented as before.

### Attachment assay using radiolabelled cells

Cells were labelled with [ $^3\text{H}$ ] thymidine at 5  $\mu\text{Ci/ml}$  for 24

8. Cells near confluence were used for the attachment assay. Cells were trypsinized and suspended in ECT. The labelled cells were collected by centrifugation and resuspended in Hanks Hepes (HH), centrifuged again and resuspended in HH. Before the cells were plated on coverslips precoated with fibronectin or serum, the cytochalasin B was added at a range of concentrations at room temperature for 15 minutes.

The cells were plated and incubated at 37 °C for 90 minutes. The coverslips were then rinsed twice in HH.

Each coverslip was transferred to a separate scintillation phial and 1 ml of 0.3 N NaOH was added and incubated for 30 minutes at 37 °C. 10 mls of water-compatible scintillation mixture (Aquasol) was added and the phials left overnight to reduce chemiluminescence. Then they were counted in the scintillation counter.

#### Iodination of fibronectin and Con A

Fibronectin was iodinated with <sup>125</sup>I as follows:

The reaction vessel was coated with iodogen (Pierce) by adding 1 mg iodogen in 1 ml chloroform and evaporating to dryness. 1 mCi Na <sup>125</sup>I in 100 µl PBS was added to 100 µl fibronectin (1 mg/ml) for 30 minutes at room temperature. Protein and free iodide were then separated on a Biogel P6 (BioRad) column.

The column was eluted with 10 ml PBS and 1 ml fractions collected. 10 µl samples of each fraction were counted on a Wilj 2001 gamma counter to determine which fraction contained fibronectin. <sup>125</sup>I fibronectin had an activity of approximately 10<sup>6</sup> cpm/ug fibronectin. This was then diluted with cold fibronectin for use.

The same procedure was used for iodination of Con A.

### Procedure for cytoskeleton extraction

1. Cell monolayers were washed twice with Hanks Hepes (HH).
2. HH with inhibitors PMSF and TAME was then added and incubated with the cells for 15 minutes.
3. HH with 1% Triton and protease inhibitors PMSF and TAME was added and incubated for 10 minutes. After the 10 minutes incubation the Triton-soluble material was removed, added to the appropriate volume of boiling mixture and analysed on SDS-PAGE gels.
4. After the removal of the Triton-soluble material, deoxyribonuclease I in Tris containing 0.5% Triton and 10 mM  $MgCl_2$  was added and left to incubate for 10 minutes.
5. After DANase treatment, the Triton-insoluble material was further extracted with 1.5 M KCl for 10 minutes.
6. The remaining insoluble material was washed with HH and diluted 1:5 with Tris-saline, added (250  $\mu$ l). Samples were boiled for 3 minutes in a water bath before being analysed on SDS-PAGE gels.

### Method for silver staining PAGE gels

Procedure used was that of Oakley et al. (1980). Due to the sensitivity of the stain, wearing of vinyl gloves is essential.

The procedure gave good staining for slab gels of 140 X 140 X 1 mm dimensions. All steps were carried out under mild agitation.

1. The gel was soaked for 30 minutes in SDS-PAGE gel destainer. Oakley et al. (1980) found that the inclusion of this step reduced the background considerably.

2. The gel was then rinse twice in double distilled water and soaked in 10% unbufferd glutaraldehyde for 30 minutes [ made from 50% biological grade glutaraldehyde (BDH Ltd)].

3. The gel was rinsed in one litre of double distilled water for 10-15 minutes, during which time the water was changed at least twice. The gel was then left overnight in 500 ml of double-distilled water.

4. The ammoniacal silver solution was made up immediately before use, as follows: 1.4 ml of  $\text{NH}_4\text{OH}$  was added to 21 ml of 0.36% NaOH. Under mild agitation 4 mls of 19.4%  $\text{AgNO}_3$  was added. A brown precipitate formed but disappeared on shaking. The volume was then made up to 100 mls with double-distilled water. A container slightly larger than the gel was used and allowed the gel to float freely in the solution. The gel was stained for 12 minutes with constant agitation. After use the ammoniacal silver solution was added to 5 mM HCl as a precaution against formation of an explosive precipitate.

5. After staining, the gel was thoroughly washed with double distilled water for 3-5 minutes.

6. Developing the stain: A solution of 0.005% citric acid and 0.019% formaldehyde [5 ml of 1% citric acid stock and 0.5 ml of 38% formaldehyde solution (BDH Ltd) in a litre] was made up before use. The proteins became visible moments after the developer was added. The gel was removed from the developer before the background became too dark.

7. Gels were kept in double distilled water until photographed for a permanent record.

#### Labelling SDS-PAGE gels with $^{125}\text{I}$ -Con A

Once completely destained the gel was placed in buffer "A" for 5-6 hours. The gel was then transferred to 250 ml of buffer "A" containing  $^{125}\text{I}$ -Con A overnight, removal from the  $^{125}\text{I}$ -Con A the gel was washed extensively for 2 days with 3 to 4 changes of buffer "A" each day. The gel was dried on a slab-drier GSD-4 (Pharmacia) and exposed to Kodak X-Omat H film with a Cronex Hi-Plus intensifying screen (Du-Pont) at  $-70^{\circ}\text{C}$  for 3-4 days before being developed.

#### Fluorescent staining of actin

NBD-Phalloidin was used as a specific fluorescent label for f-actin in spread or spreading cells. The staining procedure was similar to that of Barak et al. (1980).



The procedure was as follows: Cells were grown on coverslips and were rinsed twice in PBS. The coverslips were fixed in 4% formaldehyde in PBS for 10 minutes. Cells were washed twice in PBS and then placed in acetone at -20 °C for 5 minutes and then air dried. The cells were covered in 50  $\mu$ l of NBD-Phalloidin (in PBS) solution for 20 minutes at room temperature after which they were rinsed twice in PBS and mounted in PBS/glycerol 1:1 (vol/vol) on microscope slides.

## Chapter Three

### RESULTS

### Selection of adhesion defective mutant

I used a selection procedure in which Py3 cells were plated alternately for 18 hours in ECT and 6 hours on fibronectin in HH, using cells mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Variants which remained unattached through the selection cycles were plated in soft agar and then colonies obtained were screened for low adherence.

Variants were selected by removing the cells adhering to fibronectin. When the non-adherent cells were plated on dishes, clones of two different morphologies were found, one indistinguishable from parental cells (many spread cells), another with unspread morphology (all rounded cells) (Fig. 1). Mutants stayed in close contact, while Py3 parental cells were more scattered as though they could move. This interpretation of the colony morphology was confirmed by video-tape recording (see below). Stained mutant cells appeared to be small in size, this may be due to their complete lack of spreading.

The results of this selection were similar to those previously obtained with non-mutagenized cells (Edwards et al., 1985). No differences have been observed between the properties of recloned variants obtained in these or later selections. In order to test whether the mutant arose only once from parental cells, I recloned Py3 in soft agar and selected a third variant with the same phenotype from this clone. Subsequently, a fourth line non-adherent to fibronectin has been selected from a Tg-resistant clone (see Fig. 2). The phenotype of all isolates has proved to

Fig. 1 Clonal morphology of variant in culture

1. Upper left plate, Py3 parental cells  
right: Variant cells

Phase contrast

Scale bar: 75  $\mu\text{m}$

2. Lower left plate, Py3 parental cells

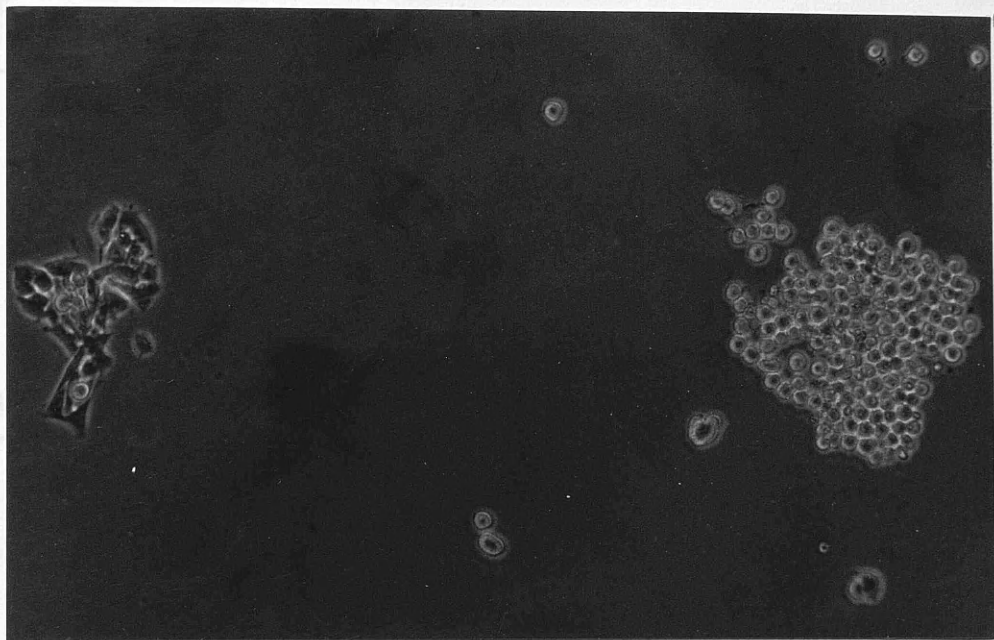
Scale bar: 50  $\mu\text{m}$

right: Variant cells

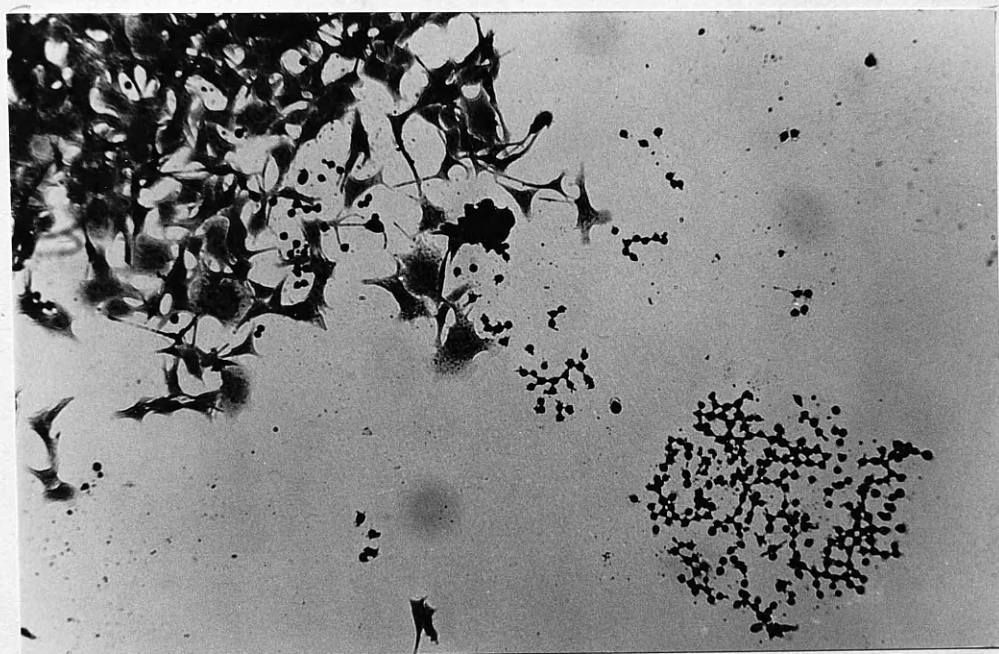
stain Coomassie blue

Scale bar: 100  $\mu\text{m}$

1

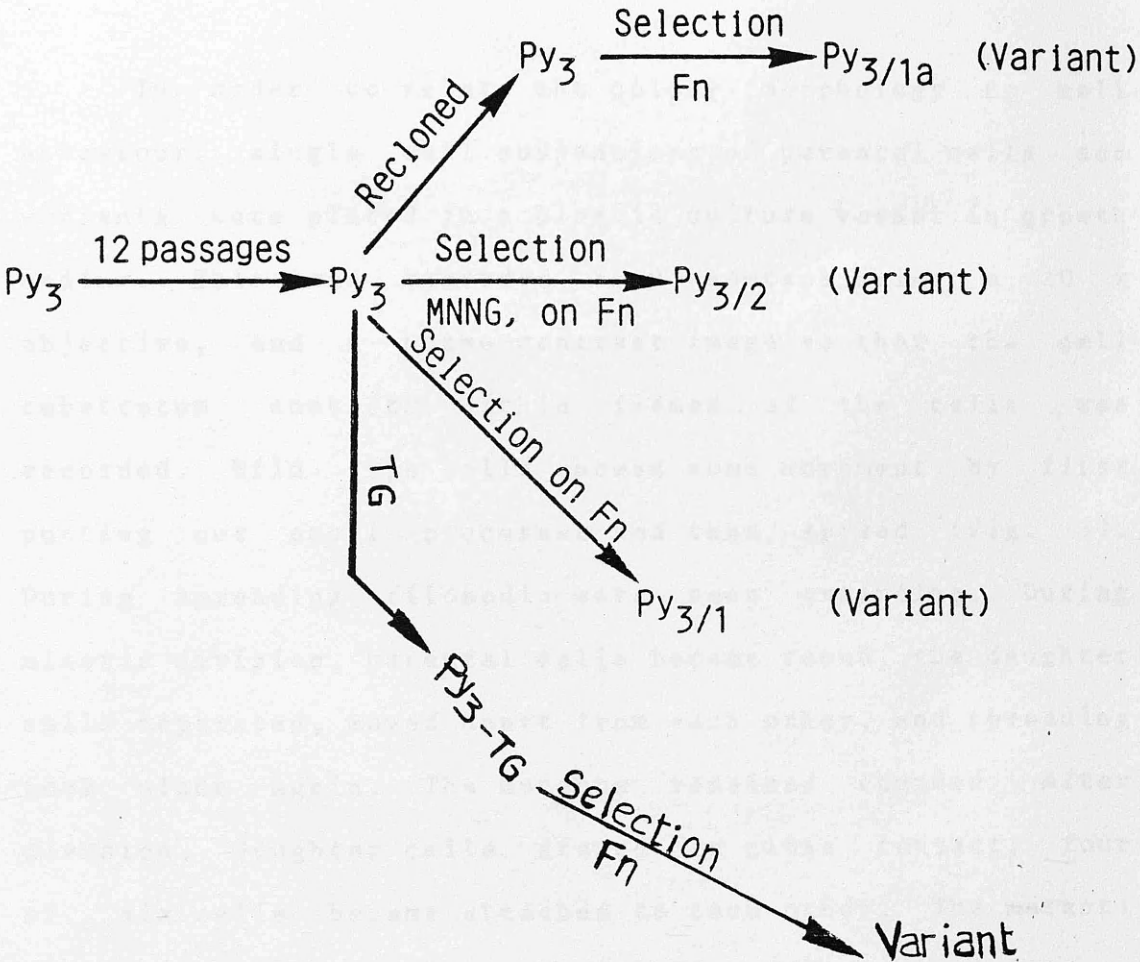


2



he stable through passage in order to  
guarantee the cells are of the same  
from their culture or a very low level of

Fig.2 Shows derivative of all the lines



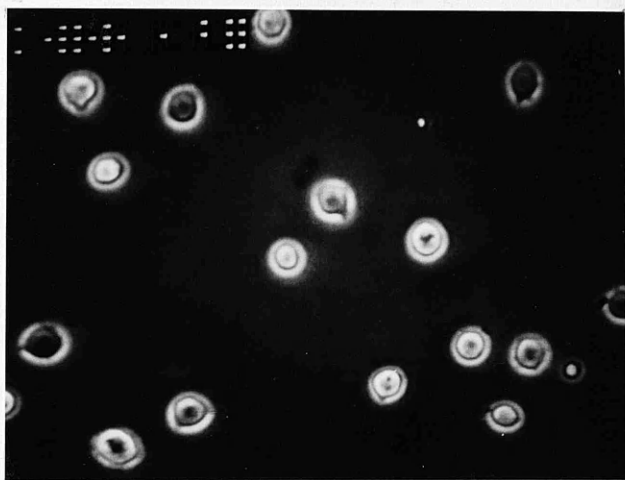
be stable through months of serial propagation under a culture regime in which cells are passed after detachment from their culture by a sharp tap (no trypsin or EDTA used) in order to minimise and eliminate the growth of revertants.

In order to relate the colony morphology to cell behaviour, single cell suspensions of parental cells and variants were plated in a plastic culture vessel in growth medium. This was recorded on video-tape using a 10 x objective, and a phase-contrast image so that the cell substratum contacts within frames of the cells was recorded. Wild type cells showed some movement by first putting out small processes and then spread (Fig. 3). During spreading filopodia were seen extending. During mitotic division, parental cells became round, the daughter cells separated, moved apart from each other, and spreading took place again. The mutants remained rounded. After division, daughter cells stayed in close contact, four or six cells became attached to each other. The mutants seemed to have the same initial ruffling activity as parental cells (Fig. 4 ).

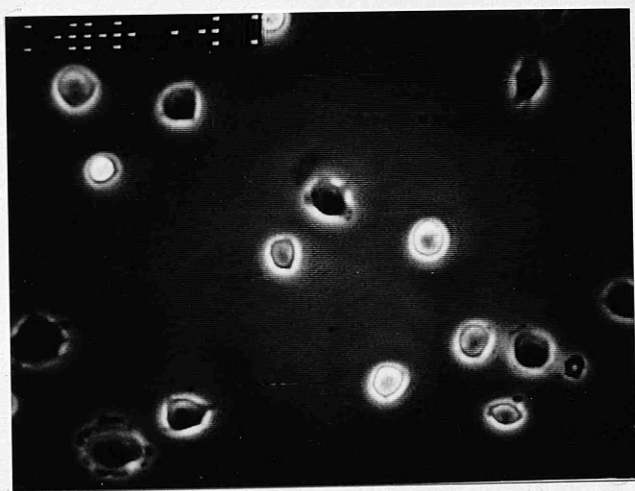
#### Chromosome number

In order to rule out the possibility that the clones of unspread morphology arose from cross-contamination of Py3 BHK with cell lines of non-adherent hybridoma cells maintained by others in this laboratory, and to test whether variants might be derived by chromosome loss from

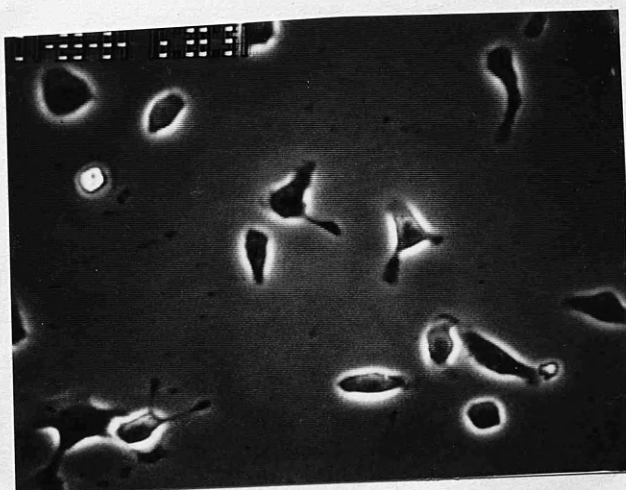
A



B



C





**Fig. 3** Video tape recording sequences of Py3  
in culture

**A. Attached cells**

**B. Flattened morphology, extending processes**

**C. Partially and fully spread cells**

**Time: 0, 1/2 hr, 2 hours**

**Scale bar: 50  $\mu$ m**

Fig. 4 Video tape recording sequence of variant  
in culture

A. Attached cells

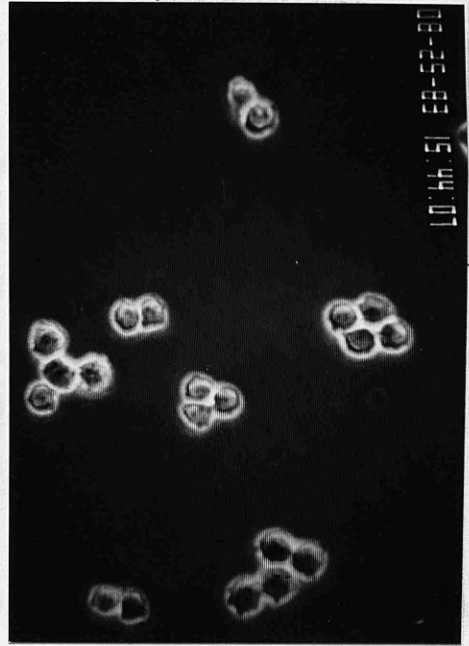
B. Cell dividing

C. Note daughter cells added

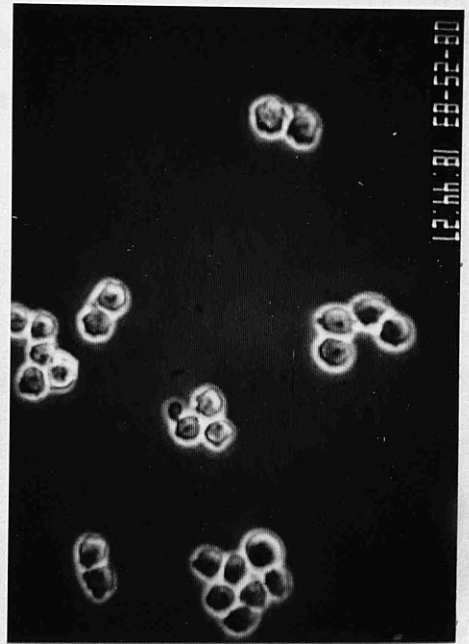
D. Small colonies formed from 5 or 8 cells

Time: 0, 2, 3, 7 hours

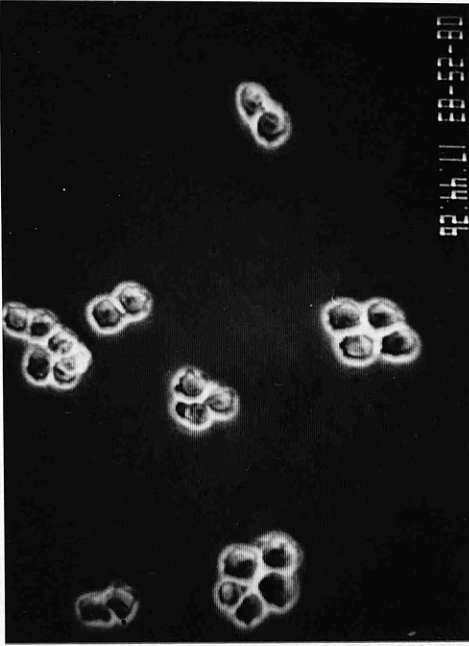
Scale bar: 75  $\mu$ m



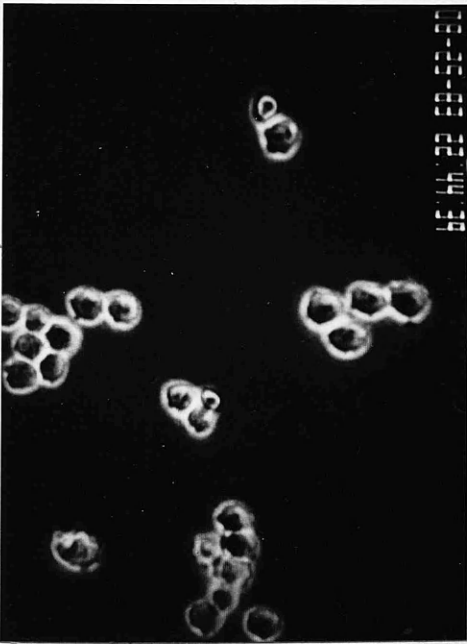
A



C



B



D

the parental cells, karyotyping was carried out. The chromosome numbers of parental, variants and mouse cells (capillary endothelium) were counted and compared. It was found that the chromosome number of variants was the same as that of parental ( about 42), the Syrian hamster diploid number is 44. The loss of chromosomes may be due to the preparation or to the difficulty to differentiate between artifacts and the small ones. The chromosomes appeared to be telocentric and metacentric. The mouse chromosome number is 40, all telocentric (Fig. 5 ). Therefore the variant is not a mouse cell.

In collaborative work with Dr M. Fried at Imperial Cancer Research Fund, the expression of polyoma T-antigens was detected in both Py3 and mutants by using antitumor sera to immunoprecipitate T-antigens from cells labelled with <sup>35</sup>S-methionine. These precipitates showed 5 identical T-antigen bands in SDS-PAGE, which confirms that mutants are derived from Py3.

#### Mycoplasma screen

Many workers reported that mycoplasmas inhibit cell metabolism, cell growth, can alter cell function and prevent cells from spreading. In order to eliminate the possibility that mutants arose from such contamination of cell lines, a mycoplasma test was carried out. The cells were stained with bisbenzimid fluorochrome (Hoechst 33258). I found that cellular DNA yielded highly fluorescent nuclei

Fig. 5 Karyotyping

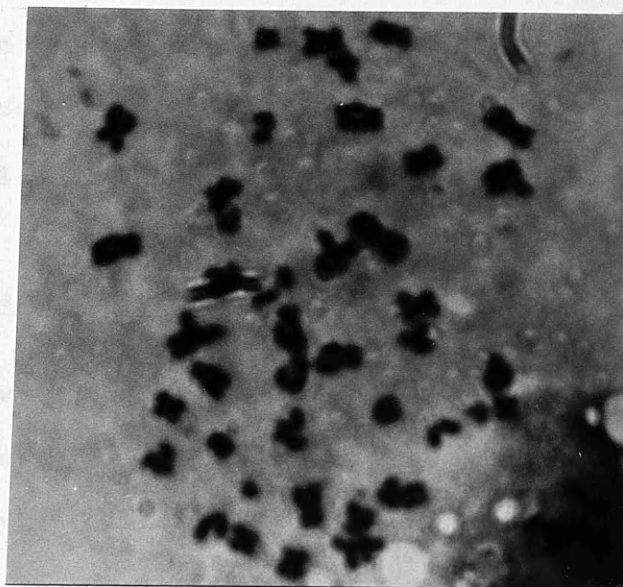
Cells were treated with colcimid, fixed and stained  
(see methods).

1. Py3 chromosome number is about 42
2. Variant chromosome number is about 42
3. mouse chromosome number is about 39

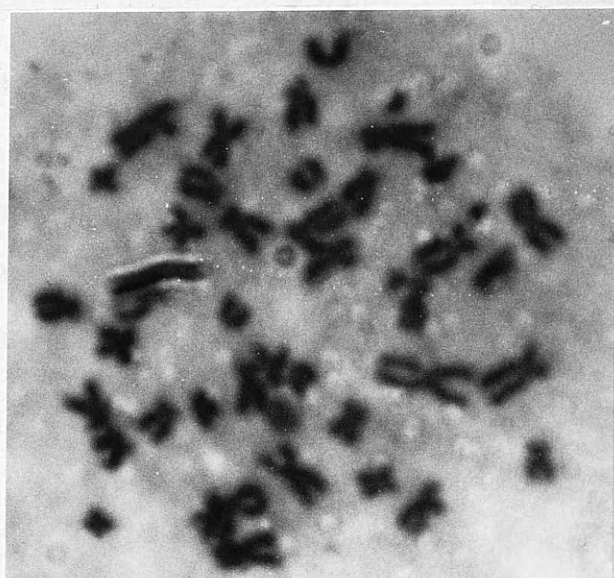
Note: ALL TELOCENTRIC

Scale bar: 17  $\mu$ m

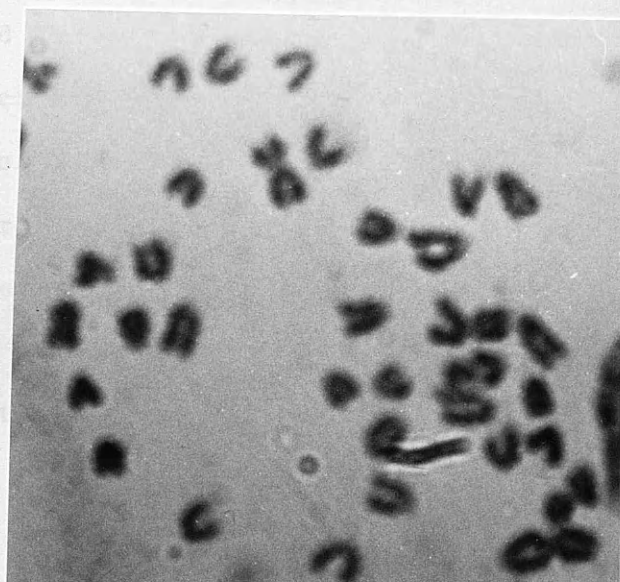
1



2



3



but no cytoplasmic fluorescence was detected. This proved that the non-adherent property of the mutant is not due to contamination (Fig. 6 ).

#### Serum spreading factor assay

Selection was carried out in 10 % calf serum which contains different adhesive proteins. Fibronectin is not the only adhesive protein in plasma or serum, another distinct protein, serum spreading factor now known as vitronectin, has been found in human and bovine sera (Barnes et al., 1980).

Serum spreading factor (vitronectin) was isolated by using adsorption to glass beads ( see methods ) (plate 1 ). The activity of the vitronectin was tested and compared to that of fibronectin (Fig. 7). It was found that spreading of parental cells increased as the concentration of vitronectin increased, just as for fibronectin. These findings disagree with the report of Knox (1984), who found that Py cells did not respond to his 70 K spreading factor from serum which was probably vitronectin. There is no marked difference in spreading of Py3 on coverslip-coated either with vitronectin or fibronectin. The mutant cells showed small processes and a very low proportion spread on both vitronectin and fibronectin, consequently the variants did not respond to vitronectin.

Fig.6 DNA nuclei (Hoechst 33258)

1. Nuclei of Py3 cells

Interphase nuclei clearly seen

Scale Bar: 20  $\mu$ m

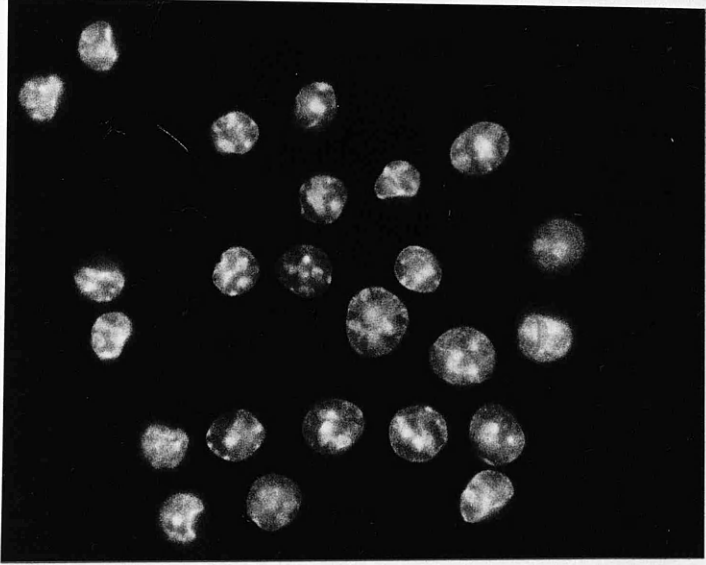
2. Nuclei of variant cells

Scale Bar: 20  $\mu$ m

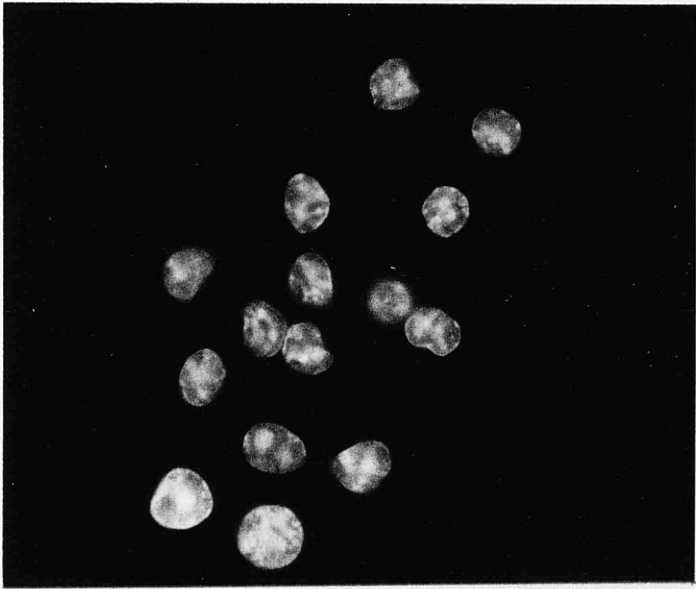
These micrographs indicate that the cultures used during selection and characterization were not contaminated with mycoplasma.



1



2



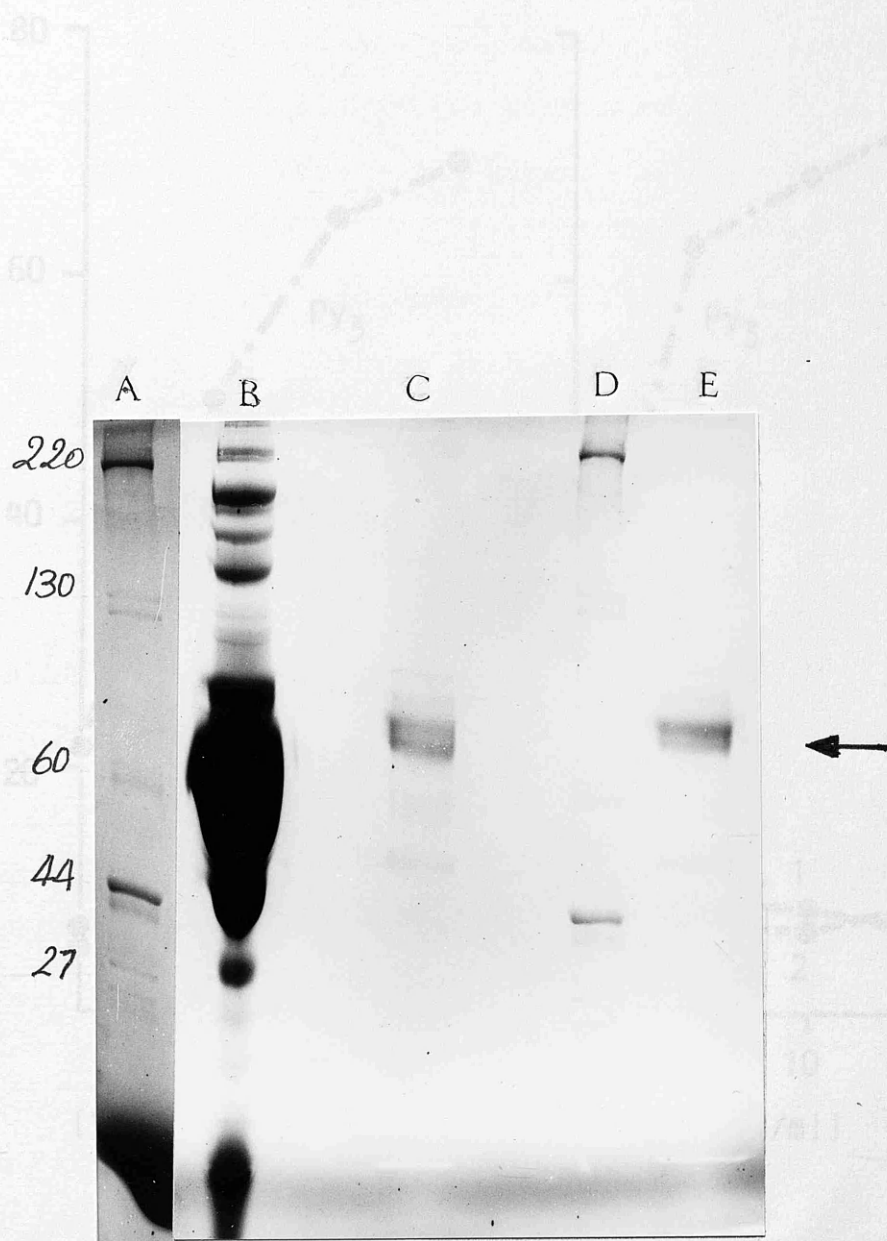


Fig. 7. Spreading assay.

Coverslips were coated either with vitronectin or fibrinogen. Cells at  $20 \times 10^5$  cells/ml were plated, were incubated for 2 hours at  $37^\circ\text{C}$ , were fixed and coverslips were then stained with Giemsa's blue. A cell spread was calculated.

Plate 1 SDS-PAGE gel

Track A standard

Track B bovine serum

Track C & E 30  $\mu$ g, 20  $\mu$ g vitronectin  
respectively

Track D 20  $\mu$ g fibronectin

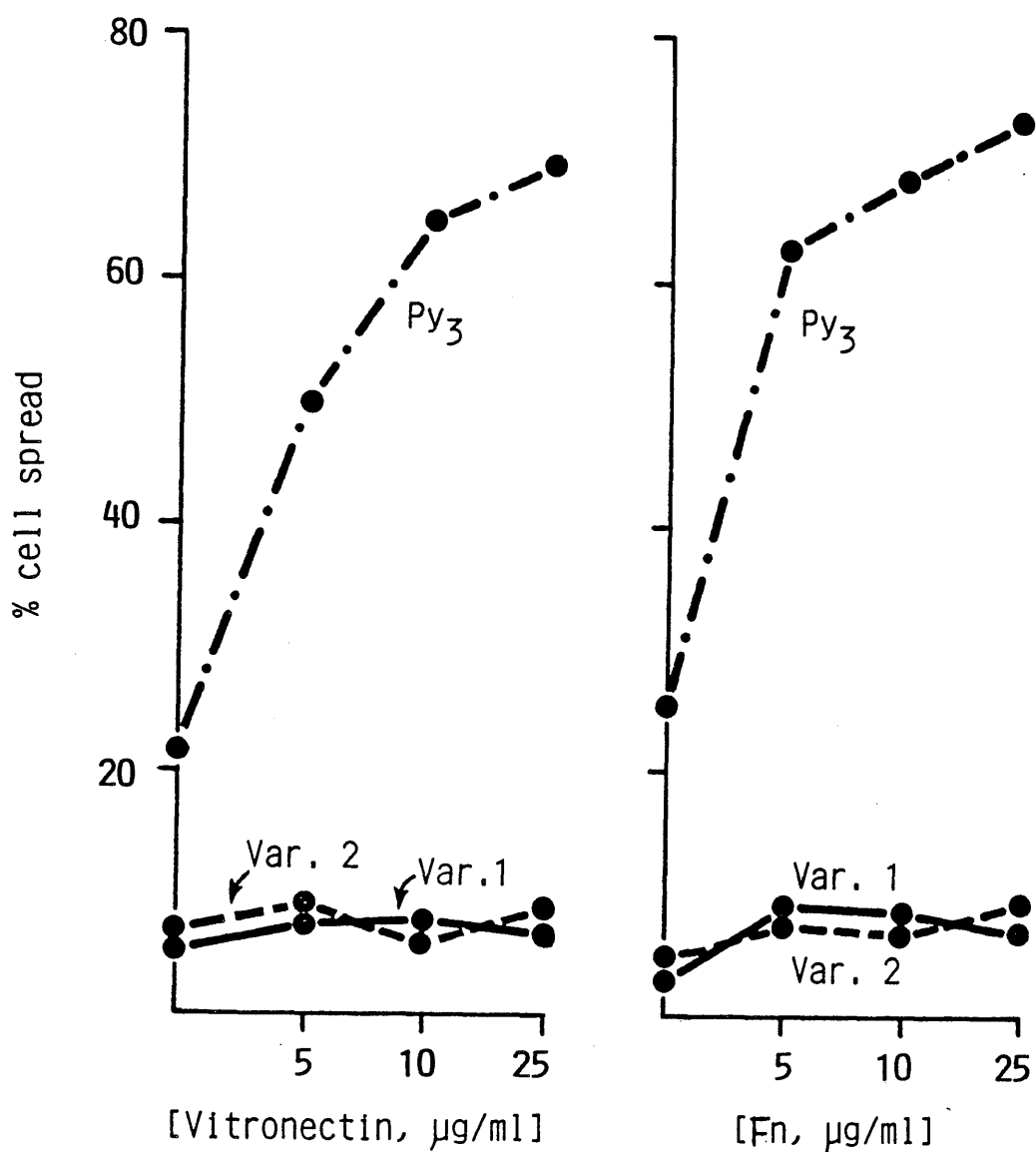


Fig. 7 Spreading assay

Coverslips were coated either with vitronectin or fibronectin. Cells at  $50 \times 10^4$  cells/ml were plated, were incubated for 2 hours at  $37^\circ\text{C}$ , were fixed and coverslips were then stained with Coomassie blue. % cell spread was calculated.

### Cell spreading assay

In order to evaluate cell-substratum spreading, Py3 and two variant cell lines were plated on coverslips coated with an adsorbed layer of serum, plasma fibronectin, vitronectin, polylysine, polyhistidine or Con A, incubated for 2 hours at 37°C, and then were fixed and stained with Coomassie blue (see methods) (Table 1, Fig. 8). Variants showed dramatically altered morphology in culture, and are unable to spread on any protein coated surfaces yet tested, or on poly-L-lysine.

Variant cells produced only a halo of fine spikes or processes, whilst parental cells were well spread. No spreading of variants beyond this stage has been observed on any protein-coated surface or clean glass, although a proportion spreads partially on tissue culture plastic (see below).

### Effect of Mn on cell spreading

It has been found by various workers that  $Mn^{2+}$  ions stimulate cell adhesion and spreading on substrates and its effect may provide a useful tool in the analysis of events involved in cell-to-substrate adhesion, cell spreading and cell motility (Rabinovitch & De Stefano, 1973; Grinnell, 1984). The effect of various concentrations of divalent manganese ions on cell spreading on clean "tissue culture treated" polystyrene plastic surface is shown in table 2. Despite the variants' failure to spread on protein-coated

Table 1. Effect of various macromolecules on the spreading of PyBHK and variant cells.

Substrate	Conc μg /ml	% cells spread		
		Py3	Var1	Var2
Serum	10%	68, 65, 60	3, 3, 6	3, 5, 3
Fibronectin	25	88, 82, 84	6, 3, 5	4, 5, 3
Vitronectin	25	77, 83, 75	4, 4, 7	4, 4, 6
Poly-lysine	10	68, 69, 65	4, 2, 5	3, 3, 5
Poly-histidine	100	84, 85, 79	3, 3, 7	4, 4, 8
Concanavalin A	50	87, 85, 80	5, 4, 7	4, 5, 9

Cells,  $5 \times 10^4$  cells/ml, were plated on precoated coverslips with proteins as indicated above. The coverslips were incubated at  $37^\circ\text{C}$  for two hours and the cell spread was scored (THREE EXPERIMENTS).

Fig. 8 Cells spreading assay

A. On fibronectin

Coverslips were coated with 25  $\mu\text{g}$  /ml fibronectin in Hanks hepes. Cells at  $5 \times 10^4$  cells /ml were plated and then were incubated for 2 hours at 37 °C. Cells were fixed and stained.

1. Py3 cells were well spread

Scale Bar: 45  $\mu\text{m}$

2. Variant 1, few cells partially spread

Scale Bar: 100  $\mu\text{m}$

3. Variant 2, note halo of fine processes.

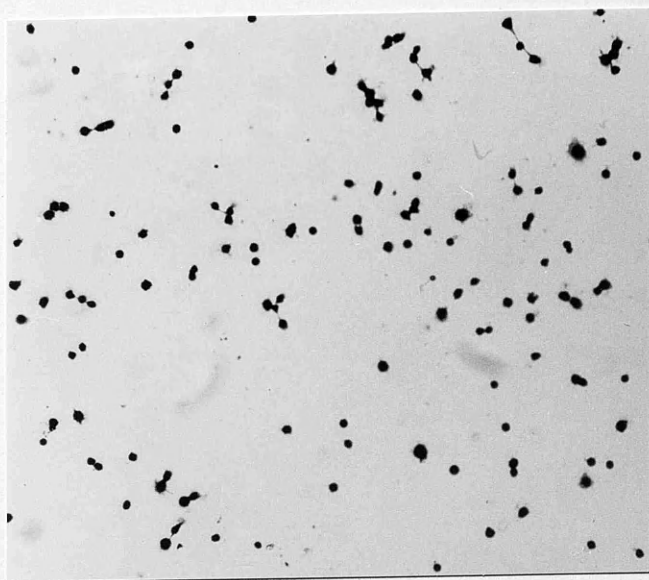
Scale Bar: 100  $\mu\text{m}$

Coomassie stain

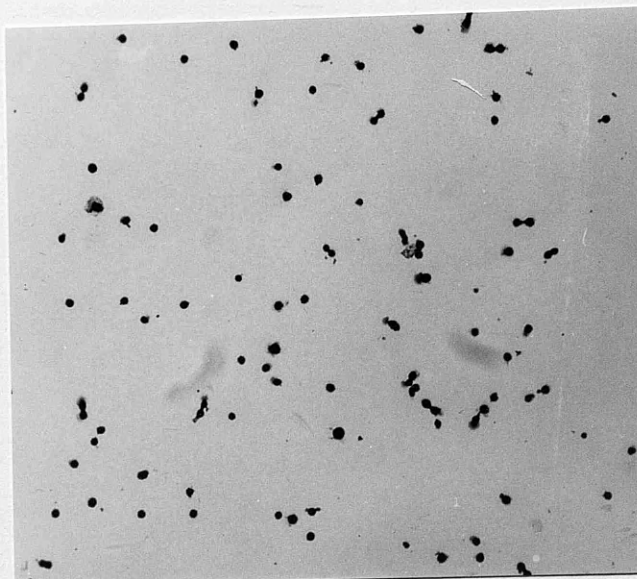
1



2



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B. On poly-L-lysine

Coverslips were coated with 10  $\mu\text{g/ml}$  poly-L-lysine in Hanks Hepes for hour and cells were then incubated for 2 hours.

1. Py3 spread

Scale Bar: 45  $\mu\text{m}$

2. Variant 1, many cells attached,  
few partially spread.

Scale Bar: 100  $\mu\text{m}$

3. Variant 2, note fine processes.

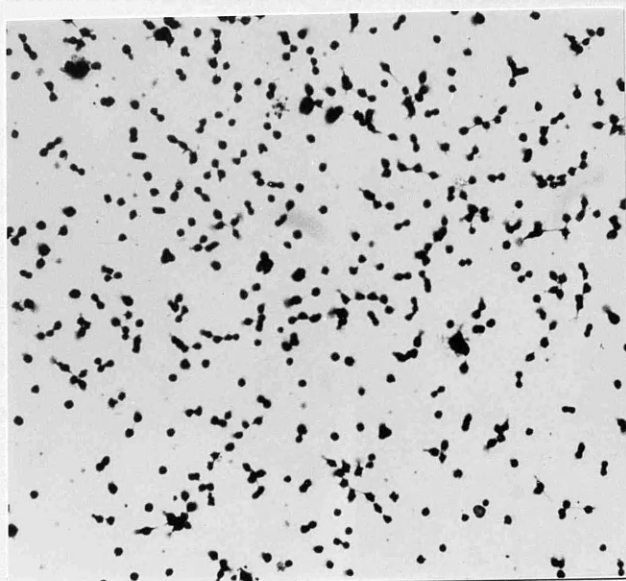
Scale Bar: 100  $\mu\text{m}$

Coomassie stain

1



2



3

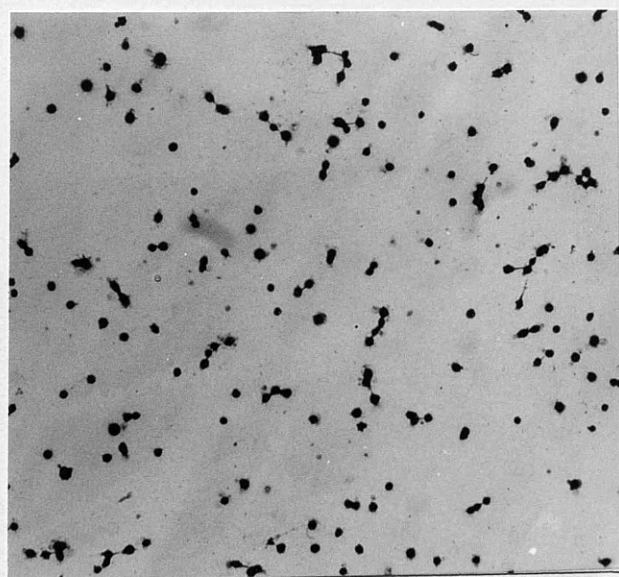


Table 2. Effect of Mn on the spreading of Py3 and variant cells on clean plastic surface.

Medium	% cells spread		
	Py3	Var1	Var2
Divalent-free	45, 50	35, 37	33, 28
10 <sup>-6</sup> M Mn	63, 58	40, 43	44, 41
10 <sup>-5</sup> M Mn	60, 65	44, 46	45, 40
10 <sup>-4</sup> M Mn	69, 63	49, 53	47, 43
10 <sup>-3</sup> M Mn	54, 50	30, 35	29, 35

Cells were scored after 90 minutes incubation (TWO EXPERIMENTS).

surfaces, a proportion of mutant cells spread on the clean plastic surface. Mn increased the percentage of spreading of wild type and there was slight increase of mutants on this surface. It appeared that, in all cases, Mn was optimally effective at  $10^{-4}$  M, while  $10^{-3}$  M was found to be inhibitory and in some cases caused visible damage to the cells.

The difference in spreading between the mutants and Py3 cells is less marked on plastic than protein-coated surfaces (Fig. 9), but is not abolished even at optimum Mn.

Py3 cells were able to attach and spread in high proportion on plastic coated with fibronectin in medium containing various concentrations of Mn, whilst only a very low proportion of mutants spread, even with optimum concentration of Mn (Table 3).

On polylysine coated tissue culture plastic, Mn increased spreading of wild type as on fibronectin, while that of mutants remained low (Table 4).

Towards the end of this work, it became possible to measure the spread area of stained cells. A digitizer interface to a micro-computer was used to monitor the degree of spreading of a number of cells. Tables 5 & 6 show the results obtained.

Fig. 9 Cell spreading assay on clean plastic  
tissue culture

A. Py3 cells spread

Scale Bar: 50  $\mu$ m

Coomassie stain

B. Variant cells, note spread cells,  
some remained rounded

Phase contrast

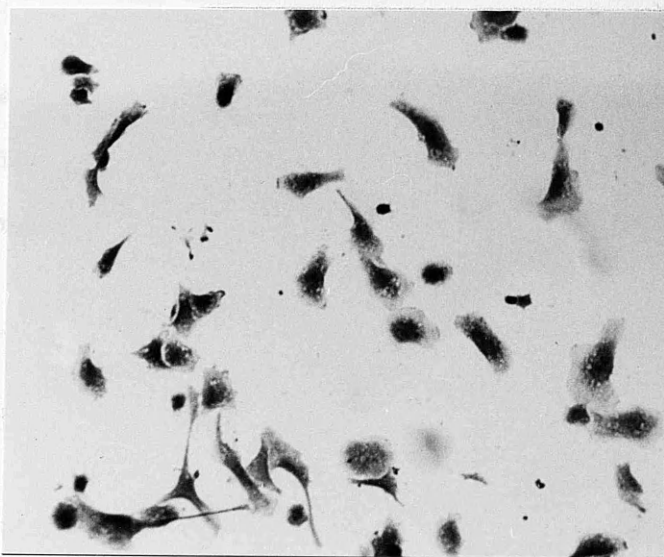
Scale Bar; 50  $\mu$ m

Table 3. Effect of Mn on the spreading of Py3 and variant cells on plastic surface coated with 25 µg /ml fibronectin.

Medium	% cells spread		
	Py3	Var1	Var2
Divalent-free	82, 84	7, 9	9, 8
10 <sup>-6</sup> M Mn	80, 78	13, 16	14, 15
10 <sup>-5</sup> M Mn	83, 81	20, 25	22, 19
10 <sup>-4</sup> M Mn	80, 84	35, 32	33, 36
10 <sup>-3</sup> M Mn	79, 73	30, 33	32, 29

Cells were scored after 90 minutes incubation  
(TWO EXPERIMENTS).

A



B

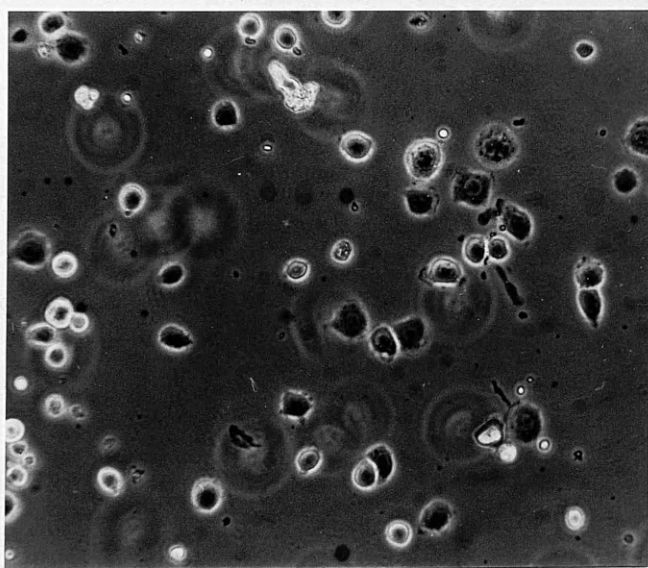


Table 4. Effect of Mn on the spreading of Py3 and variant cells on plastic surface coated with poly-lysine ( 25  $\mu$ g /ml).

Medium	% cells spread		
	Py3	Var1	Var2
Divalent-free	68, 64	6, 4	5, 6
$10^{-6}$ M Mn	58, 56	15, 17	14, 16
$10^{-5}$ M Mn	65, 63	30, 34	32, 36
$10^{-4}$ M Mn	76, 78	40, 38	35, 39
$10^{-3}$ M Mn	62, 60	36, 38	34, 32

Cells were scored after 90 minutes (TWO EXPERIMENTS).



Table 5. Effect of Mn on cell areas

Glass surface	Mean spread area	
	Py3	Variant
Divalent free	141.3	39.5
$10^{-4}$ M Mn	93.9	42.1
Fibronectin + $10^{-4}$ M Mn	169.6	40.3

\* Picture cells at x25.

Table 6. Effect of Mn on cell areas

Plastic surface	Mean Spread Area	
	Py3	Variant
Divalent free	33	26.4
$10^{-4}$ M Mn	26.3	22.4
Fibronectin (25 $\mu$ g /ml)	35.9	19.2
Fibronectin + $10^{-4}$ M Mn	35.1	21.8

\* Picture cells at x10.

On clean glass, where no divalent manganese ions were added, parental cells were spread while the mutants produced only processes. On the same surface, Mn ions at  $10^{-4}$  M did not increase the spread area of wild type cells, spread area decreased was due to elongation, while the mutant showed a slight increase.

On fibronectin-coated glass, the spread area of wild type cells increased in presence of Mn, while that of mutants showed no increase (Table 5).

On tissue culture grade polystyrene, where no divalent was added, Py3 cells were spread while the mutants showed a proportion of spreading. On the same surface, Mn ions at  $10^{-4}$  M did not increase spread area of wild type cells, while the mutant did not show an increase in spread area. On fibronectin-coated plastic, no increase in spread area of wild type was observed in presence of Mn, while the spreading area of mutants remained low (Table 6).

#### Adhesion under flow condition

In this assay a cell is allowed only the brief lifetime of its collision with the surface to establish adhesion. A fluid stream through the flow chamber was achieved with a constant pressure device that eliminates pulsatile flow. Under this condition, the flow should be laminar. The reason for using this assay to minimize the contribution to adhesion of spreading and motility-related activities of the cells.

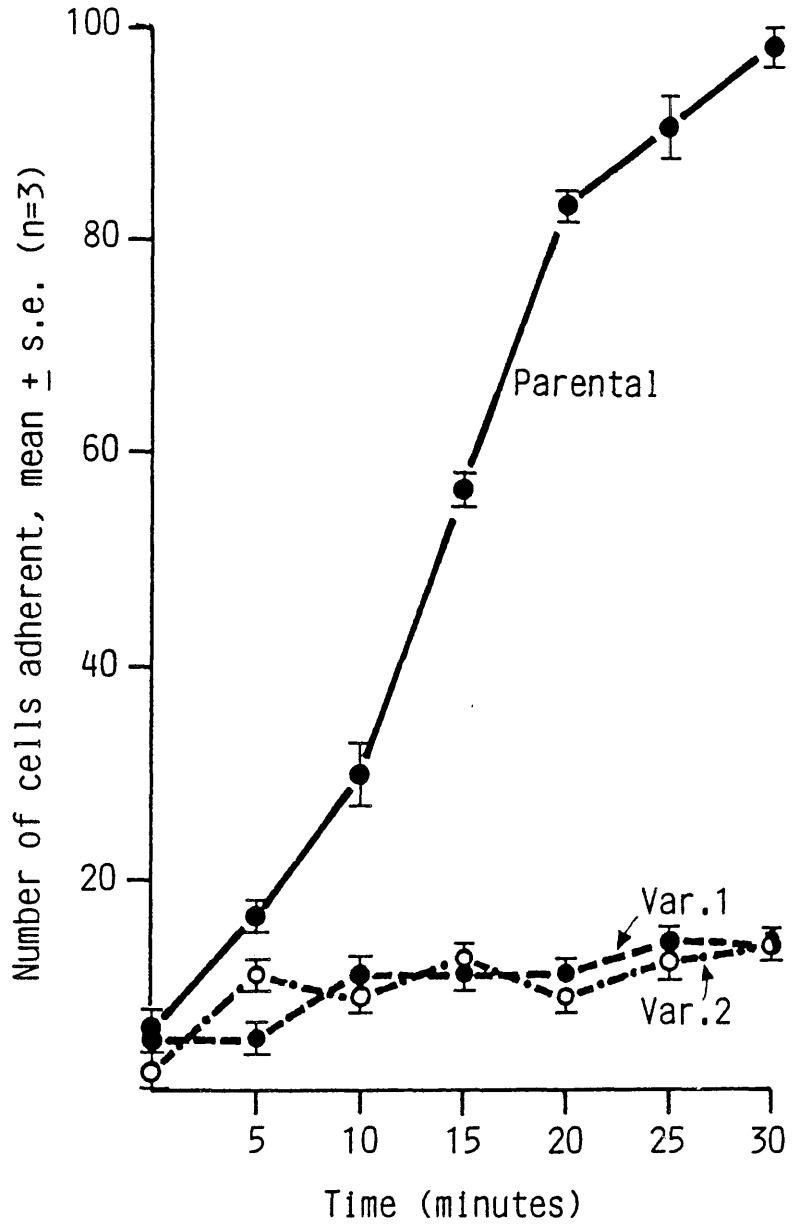
The cell suspensions were kept on ice and were agitated before being used. Monodisperse suspended cells were perfused through the chamber and the kinetics of attachment to the lower surface of the flow chamber were determined as described in Materials and Methods.

Very marked differences were found between variants and parental lines in adhesion to fibronectin (6 fold) (Fig. 10 ) to serum (3 fold) (Fig. 11 ), and to fibronectin depleted serum (3 fold) (Fig. 12 ). However all three lines adhered with equal probability to poly-lysine (Fig. 13 ), even when poly-lysine was diluted with BSA (100 mg/ml) (Fig. 14 ) or the flow rate increased (Fig. 15). The variants therefore adhere well to one of the surfaces (polylysine) on which they are unable to spread.

Py3 cells were rarely seen to roll along the chamber wall on any substrates. Contact seemed always to lead to an adhesion, although there were possibly a few more cells rolling on serum than fibronectin. Initially Py3 cells on coated surfaces were rounded, but after 20 minutes they started to flatten, although as expected, the mutant on polylysine remained rounded. A few variants cells rolled along the chamber wall at slow flow rate.

It might be expected that attachment in the flow chamber would be independent of cytoskeletal events. To test this, wild type cells, Py3, were treated with inhibitors such as cytochalasin or EHNA (see materials and methods),

Fig.10 Adhesion of Py<sub>3</sub> and variant cells in F. chamber coated with fibronectin (25µg/ml)



Monodisperse suspensions of Py<sub>3</sub> or variant cells in HH at  $0.5 \times 10^6$  cells/ ml were passed through the chamber at rate of 0.02 mls / min.

Fig.11 Adhesion of Py<sub>3</sub> and variant cells in flow chamber coated with 10% serum

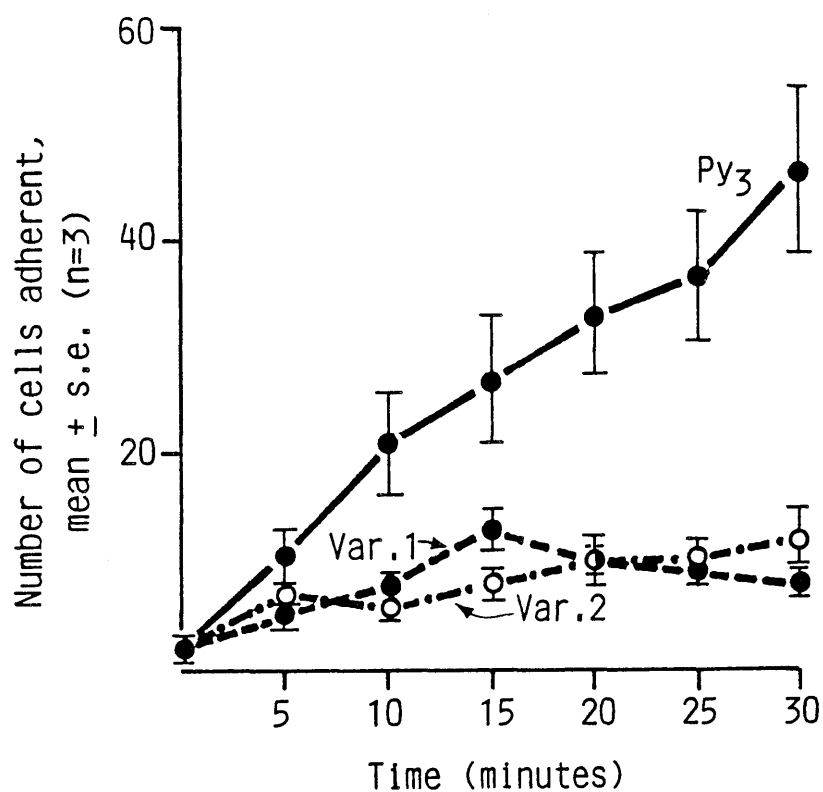


Fig.12 Adhesion of Py<sub>3</sub> and variant cells  
in flow chamber-coated with  
fibronectin depleted serum

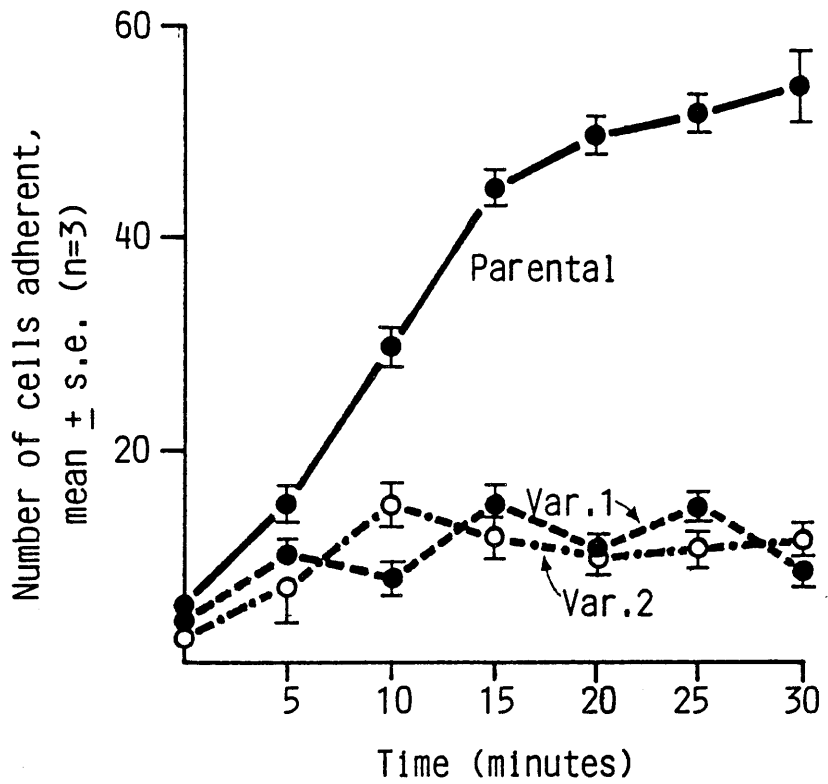


Fig.13 Adhesion of Py<sub>3</sub> and variant cells in flow chamber coated with poly-lysine (25μg/ml)

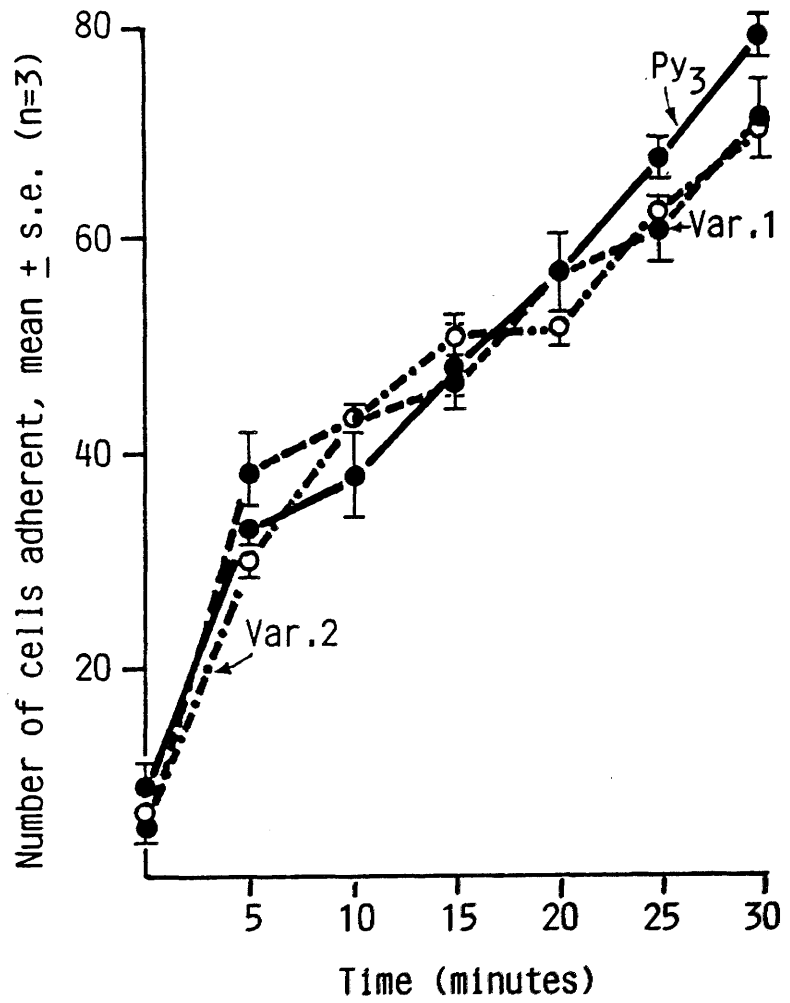


Fig.14 Adhesion of parental and variant cells in flow chamber coated with poly-lysine diluted with BSA

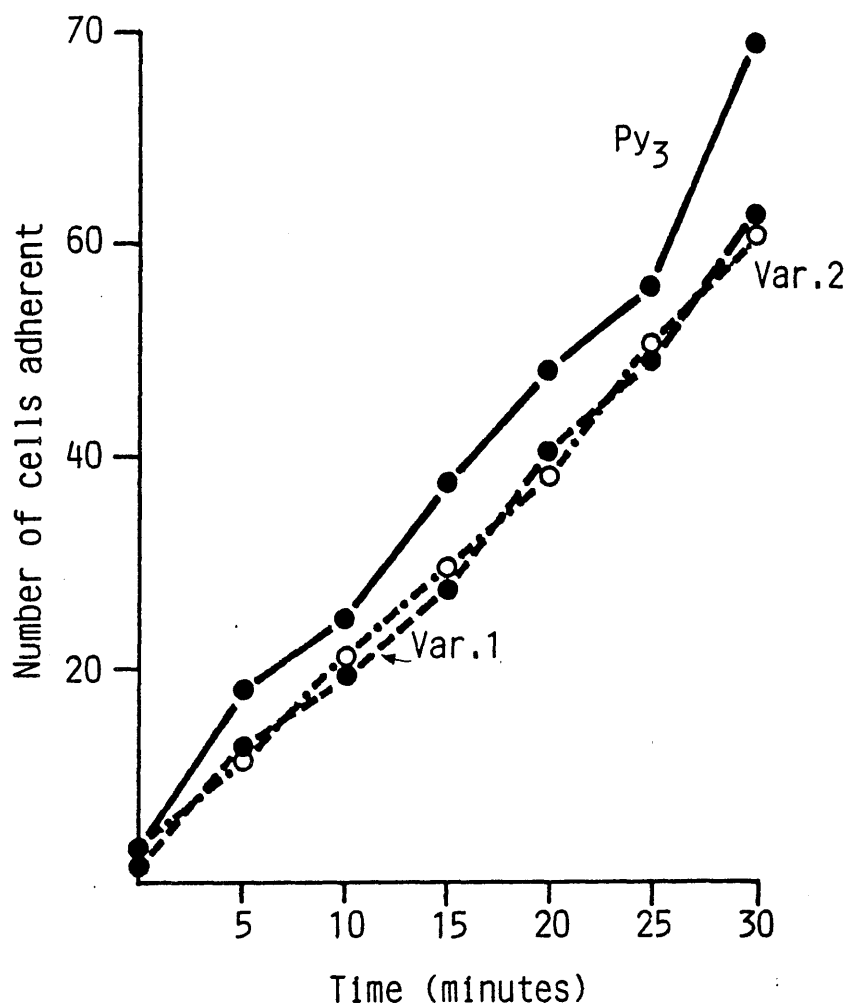
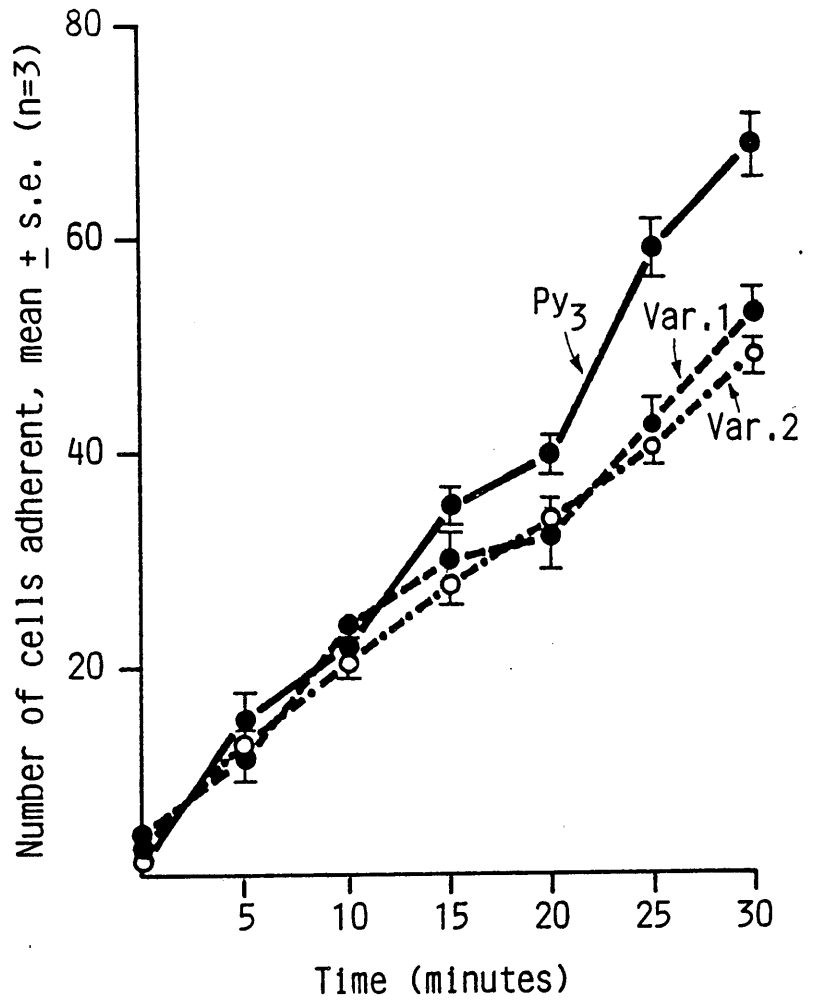




Fig.15 Adhesion of Py<sub>3</sub> and variant cells in flow chamber coated with poly-lysine (25μg/ml) when flow rate increased



and their ability to adhere to surface coated with serum or fibronectin in a flow chamber was compared. It was found that the inhibitors decreased adhesion in the flow chamber by almost 50% in comparison to untreated cells (Fig. 16 ). This was not the expected result and is discussed further below.

#### Cell attachment assay

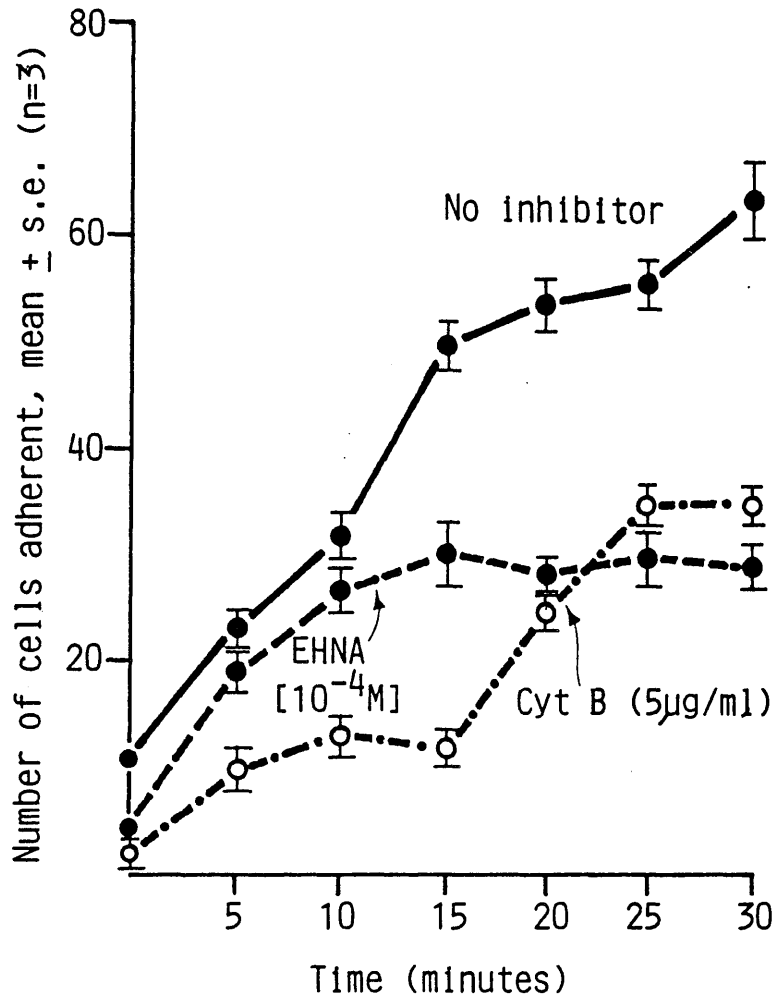
A conventional (static) adhesion assay was carried out in order to test the sensitivity of adhesion to inhibition by cytochalasin B. Labelled parental cells, Py3, were treated with cytochalasin B at various concentrations. The radioactivity associated with the adhering Py3 cells was determined as described in Materials and Methods.

The ability of Py3 cells to adhere to surfaces coated with serum or fibronectin was compared. It was found that the extent of adhesion was decreased as the cytochalasin was increased (Fig. 17 ), the maximum inhibition was about 70%. These results were therefore similar to those obtained in the flow chamber adhesion assay.

#### Test of cytoskeletal activity: FITC Con A capping

There is no single assay which might allow one to test the functional status of all components of the cytoskeleton. However, lectin induced capping is a complex cytoskeletal activity that requires coordination of both microfilaments and microtubules (Nicholson, 1976). Thus the

Fig.(16a) Effect of inhibitors on adhesion of Py<sub>3</sub> in F.chamber-coated with 10% serum



Morodisperse suspensions of Py<sub>3</sub> cells at  $0.5 \times 10^6$  cells/ ml were treated with  $5 \mu\text{g}$  /ml cytcchalasin B or  $10^{-4}$  M EHNA for 15 minutes at room temperature. Treated cells were passed through the flow chamber as well as untreated cells.

Fig.(16b) Effect of inhibitors on adhesion of  
Py<sub>3</sub> in F.chamber-coated with Fn  
(25μg/ml)

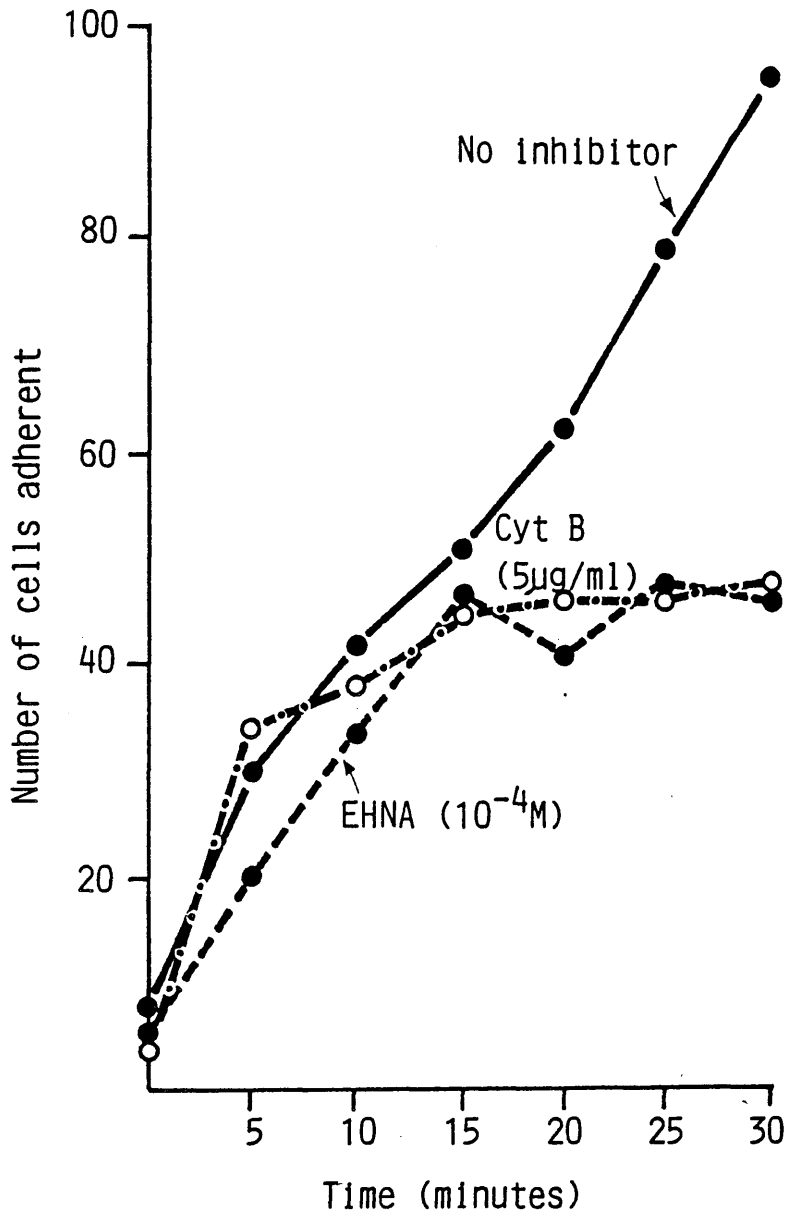
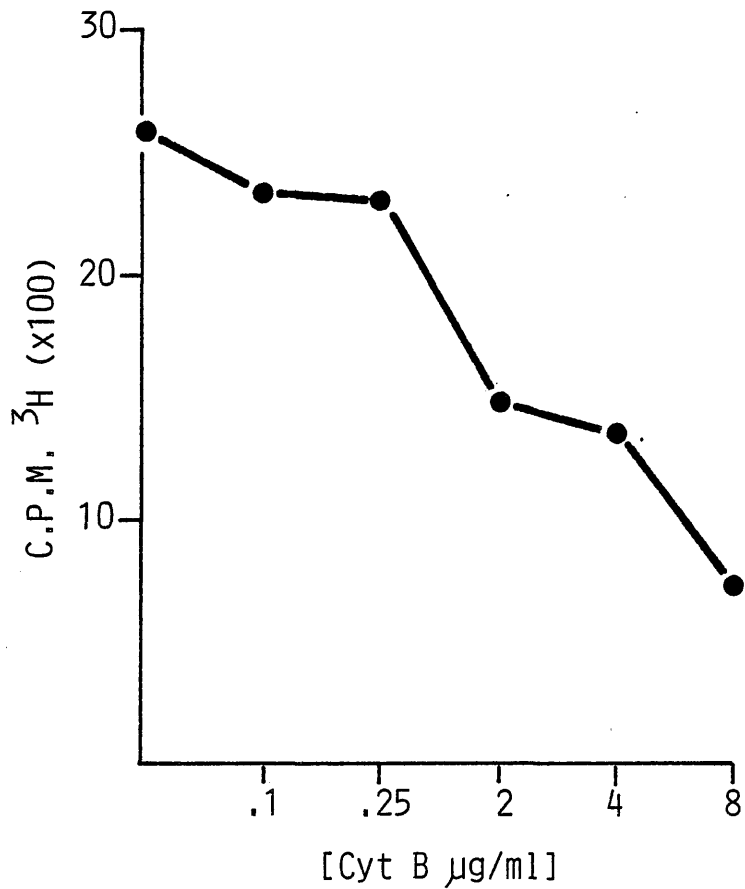
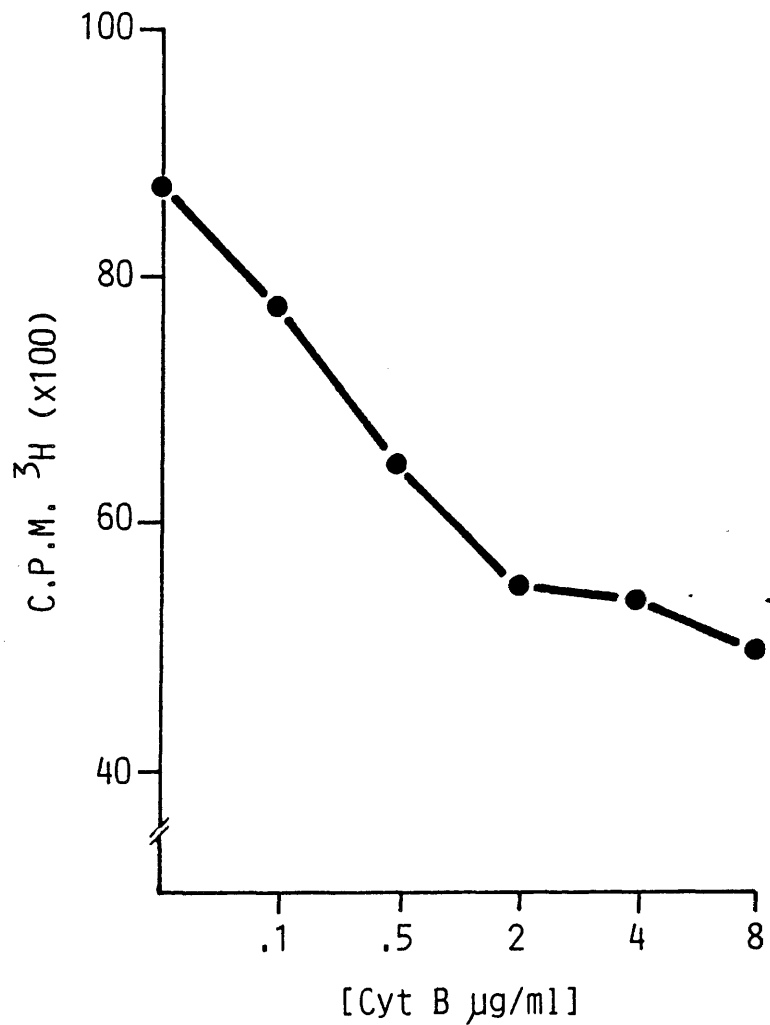


Fig.(17a) Effect of Cyt B on adhesion of Py<sub>3</sub>  
on coverslip coated with 10% serum



Py<sub>3</sub> cells were labelled with [<sup>3</sup>H] thymidine at 5 µCi/ml. Cells were then treated with cytochalasin B at a range of concentrations, were plated on coverslips coated with 10% serum and were incubated at 37°C for 90 minutes. The radioactivity was counted ( see methods).

Fig.(7b) Effect of Cyt B on adhesion of Py<sub>3</sub> on coverslip coated with fibronectin (25µg/ml)



Py<sub>3</sub> cells were labelled with [<sup>3</sup>H] thymidine at 5 µCi /ml. Cells at 5 x10<sup>4</sup> cells/ml were treated with cytochasin B at a range of concentrations, were plated on coverslips coated with 25 µg/ml fibronectin and were incubated at 37 °C for 90 minutes. The radioactivity was counted as described in methods.

ability of a cell to cap a lectin would seem a good indication of the functionality of some of its most important cytoskeletal components.

When the ability of Py3 and variants to cap FITC Con A was compared, it was found that both Py3 and variant cells could form caps (Plate 2 ). The extent of capping was quite similar in Py3 and variant cells. Thus no difference was found between parental and mutants by using this assay.

#### Scanning electron microscope observations

The initial contacts between cells and substratum are believed to be made by numerous filopodia followed presumably by activation of the cellular contractile system (Heath & Dunn, 1978) to flatten down the cell onto the substratum. SEM provides a more effective means of visualizing the detailed cells folding of the surfaces than the light microscope.

Py3 and variant cells were seeded on coverslips coated with poly-L-lysine (see Materials & Methods). Following initial attachment and spreading after 30, 60, 90 minutes incubation, Py3 cells exhibit a flattened appearance with little blebs. In plate (3) Py3 cells in various stage of spreading may be seen and those in the early stages of the process possess many filopodia. Variant cells (Plate 4 ) were blebbed, their surfaces were covered with microvilli and in one case a small process resembling

Plate 2 FITC-Con A capping

Cells were treated with 50  $\mu\text{g m/}$  FITC-Con A in PBS for 15 minutes at 4 °C, after which the lectin-treated samples were transferred to 37 °C for 60 minutes (see methods).

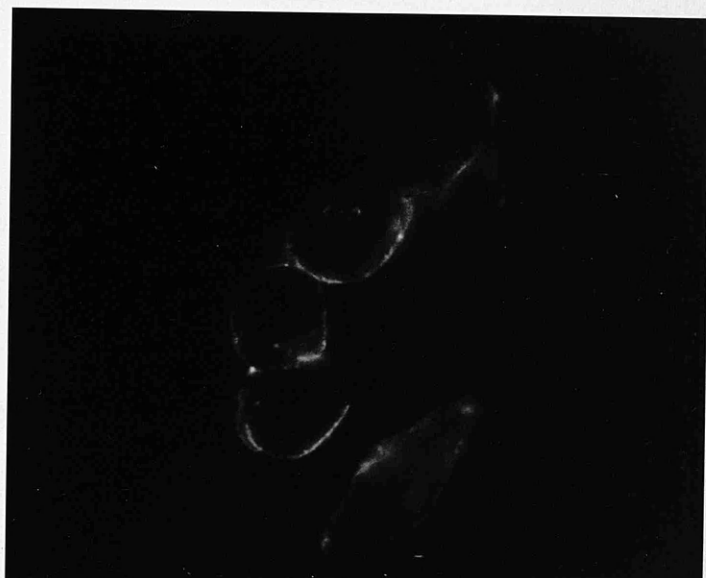
Plate 2A Py3 cells

1. Patching, note spread cell, after 30 minutes
2. Caps, after 60 minutes

Scale Bar: 50  $\mu\text{m}$



1



2

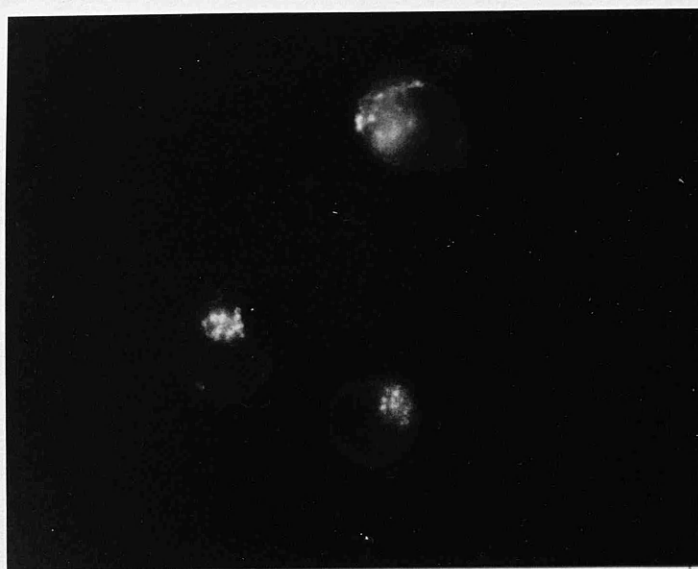
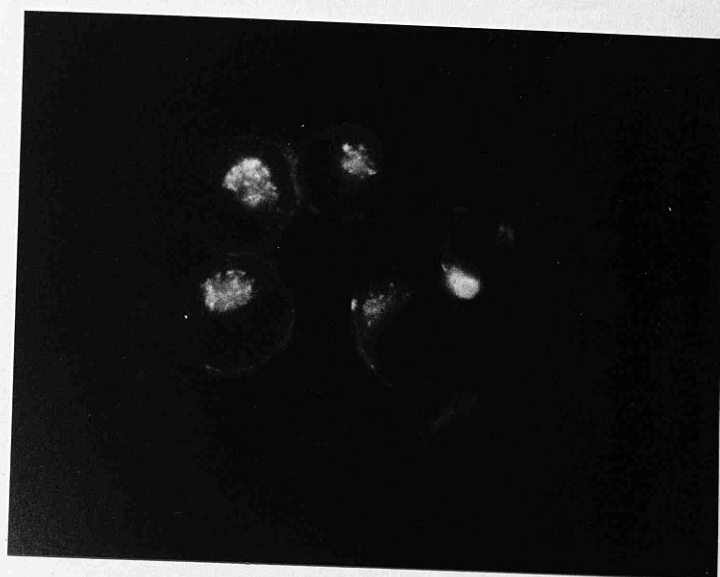


Plate 2B Variant 1

Caps clearly seen,  
after 60 minutes.

Scale Bar: 50  $\mu$ m



1



2

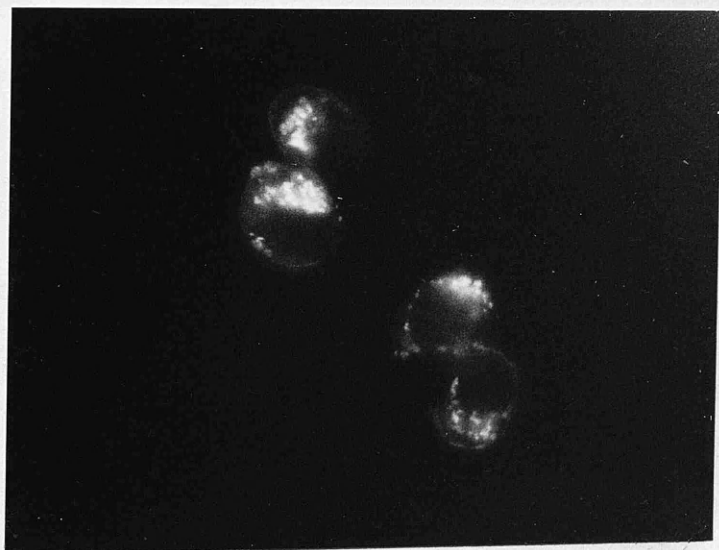
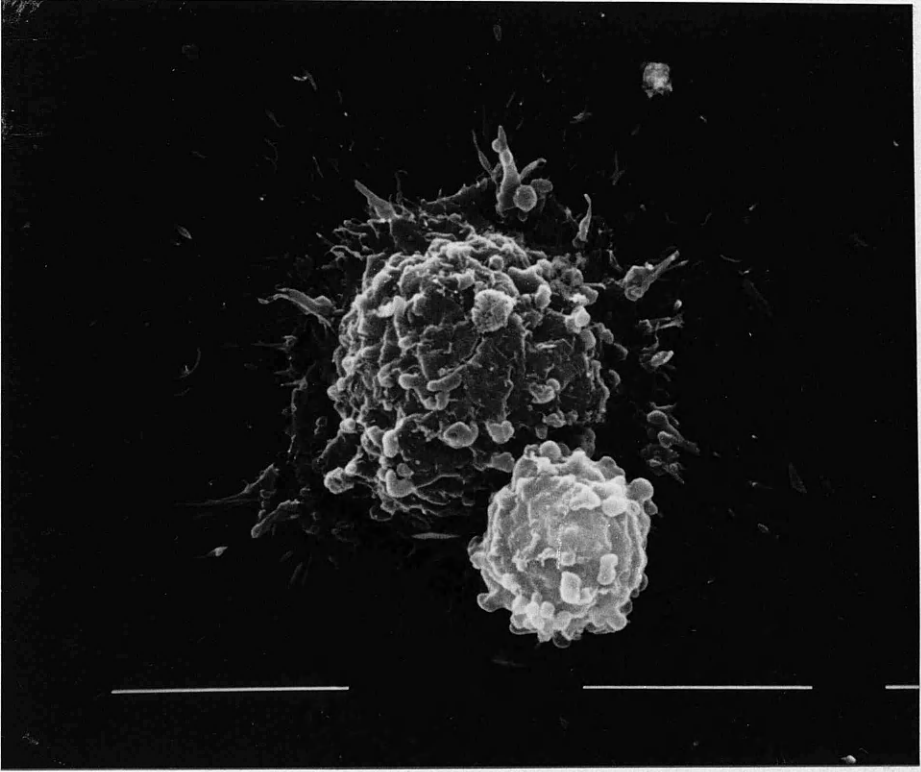


Plate 3     Scanning micrographs of Py3 cells

1. Note the flattened appearance of Py3 cells, and blebbed rounded cell.
  2. Flattened appearance of the spread cell, Note a dead cell. Surface cracking due to damage from critical point drying.
- Scale bar: 10  $\mu\text{m}$

1



2

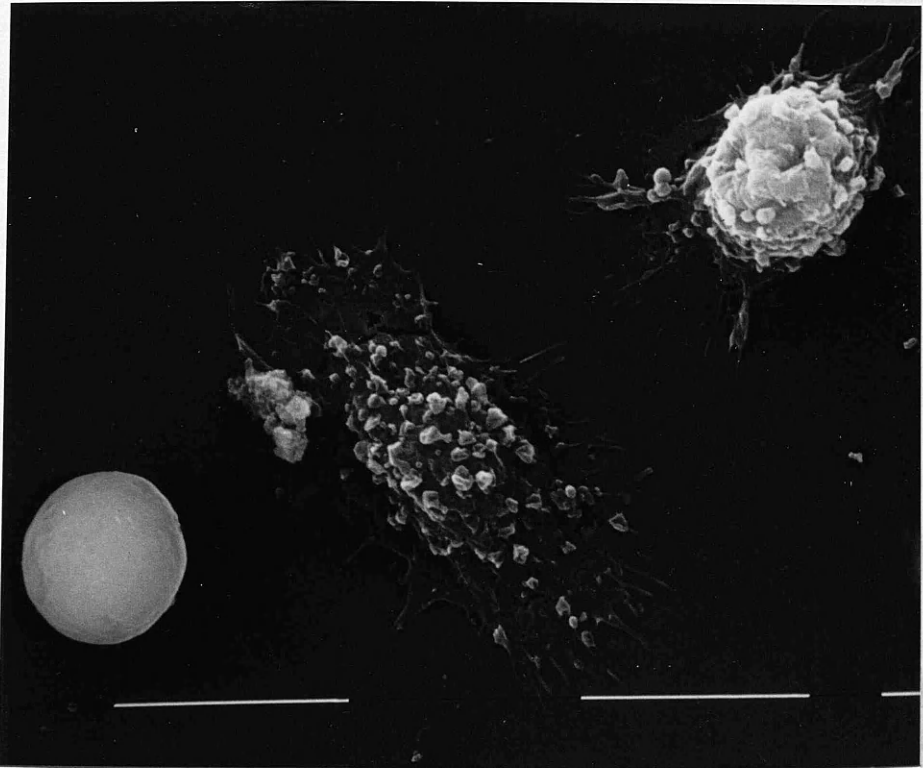


Plate 4 SEM micrographs of Variants

Plate 4A Variant 1

Blebbed and microvillous rounded cells.

Note the process.

Scale bar: 10  $\mu\text{m}$



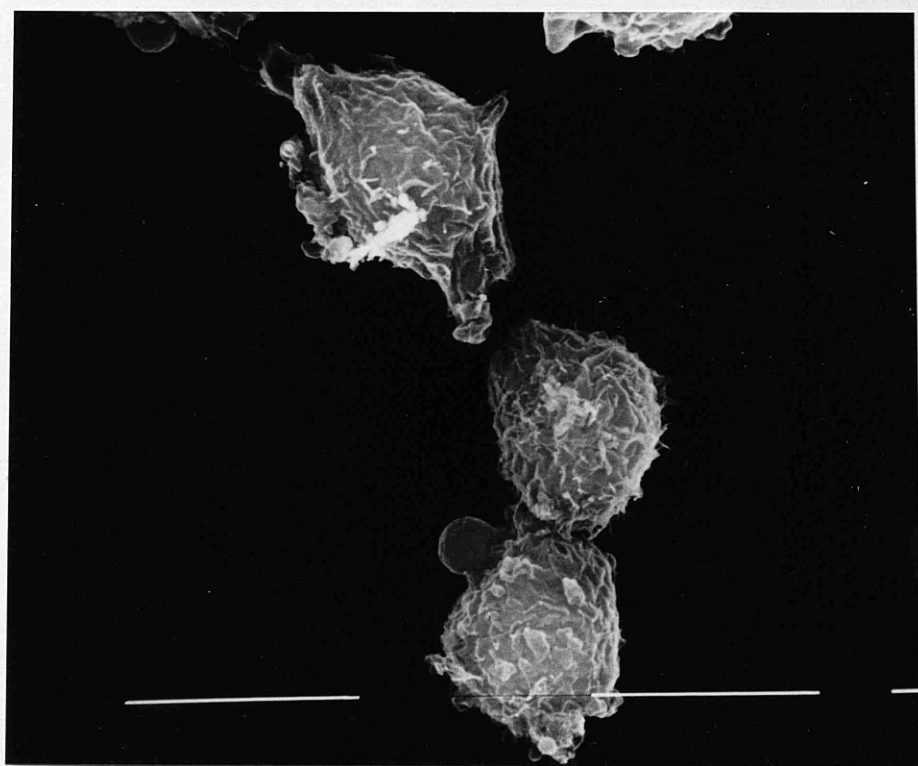
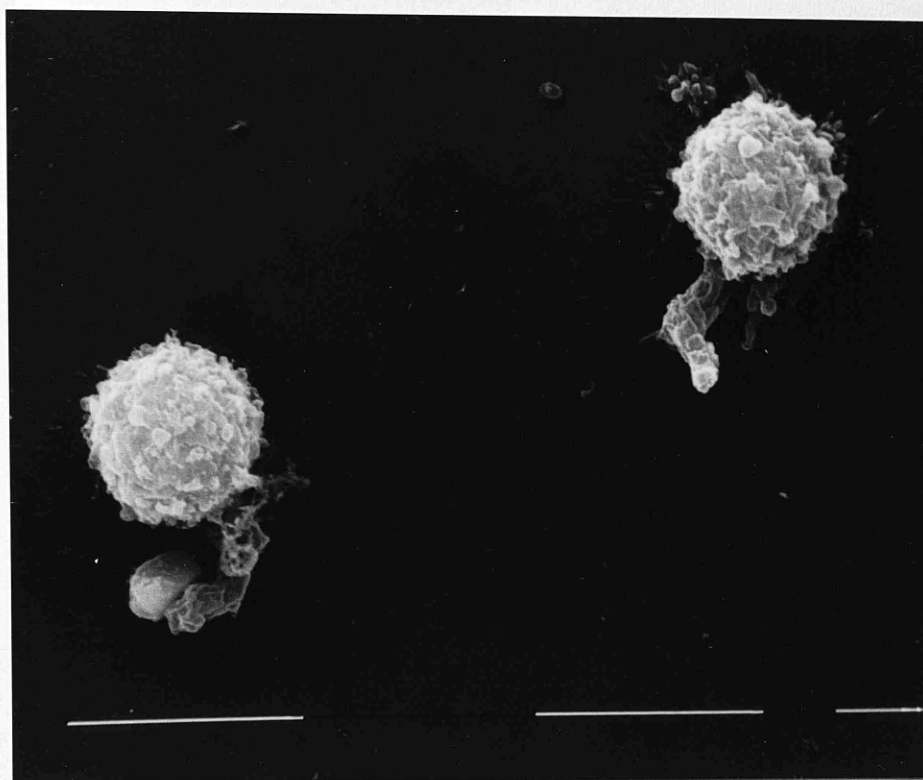
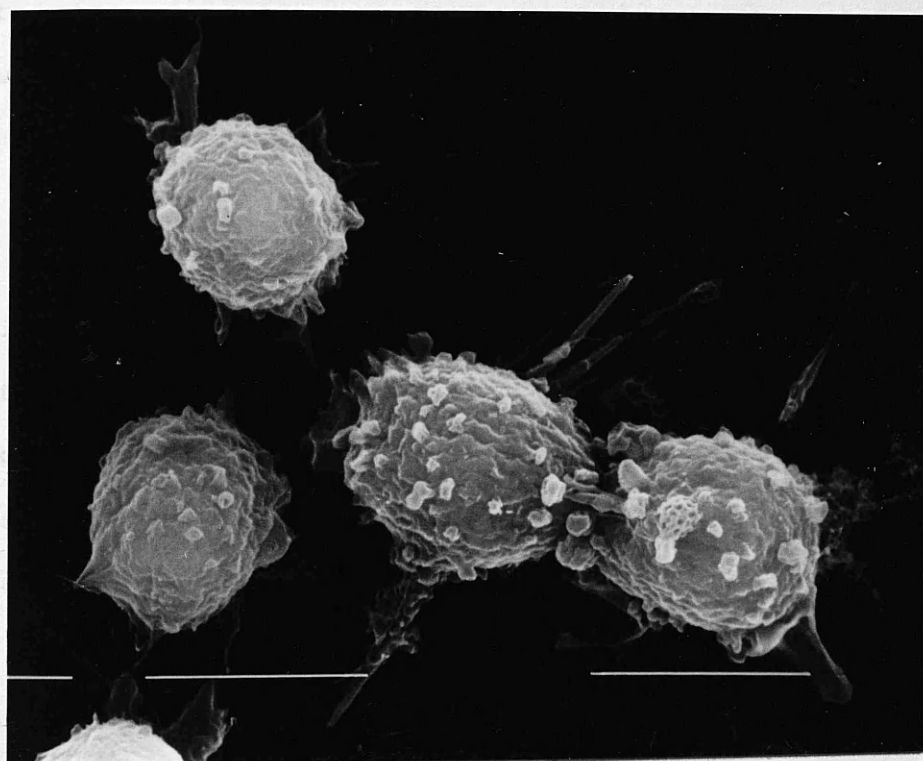
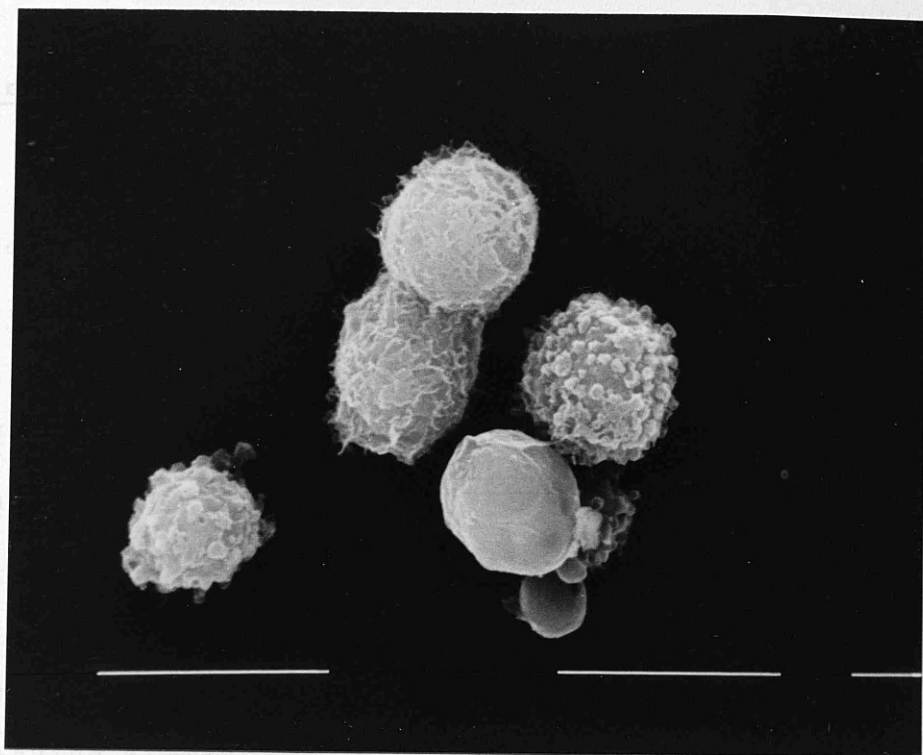




Plate 4E Variant 2

Remained rounded, extending fine spikes,  
microvillous cells.

Scale bar: 10  $\mu\text{m}$



an uropod was extended.

### Distribution of f-actin during cell spreading on fibronectin

The localization of actin during cell spreading was detected using NBD-Phalloidin, a specific fluorescent label for f-actin (Barak et al., 1980). Py3 cells gave a similar staining pattern to that described by Lazarides (1975) and Connolly et al. (1981), developing stress fibers when fully spread (Plate 5 ).

During spreading in the presence of fibronectin, I observed that many fully spread single cells , in addition to having stress fibers, showed short hair-like protrusions of the cell edges. I assume that these are f-actin-containing filopodia (Plate 5) because initial contacts between cells and substratum made by filopodia followed by the cellular contractile system.

Variant cells formed a fluorescent ring, fluorescent stain also accumulated in contact areas of cells and even in those which developed processes or partially spread. No filopodia were seen in these cells. I assume f-actin is present underneath the plasma membrane of rounded cells and at cell-cell contact areas (Plate 6 ).

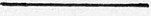
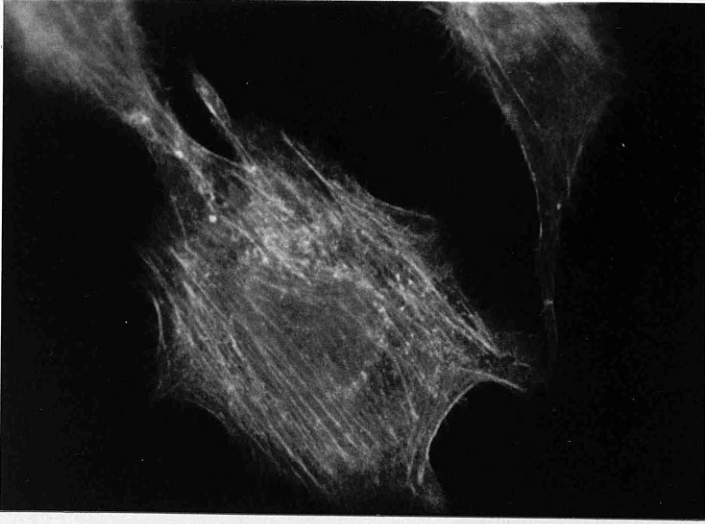
Plate 5 Actin distribution in Py3 cells

A. Stress fibres display in Py3,  
cells spread on fibronectin.

B. Note short hair like processes

Scale Bar: 20  $\mu\text{m}$

A



B

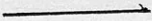


Plate 6 Actin distribution in Variants

Plate 6A Variant 1

A fluorescent ring developed.

Note actin stain in contact areas.

Scale Bar: 50  $\mu\text{m}$

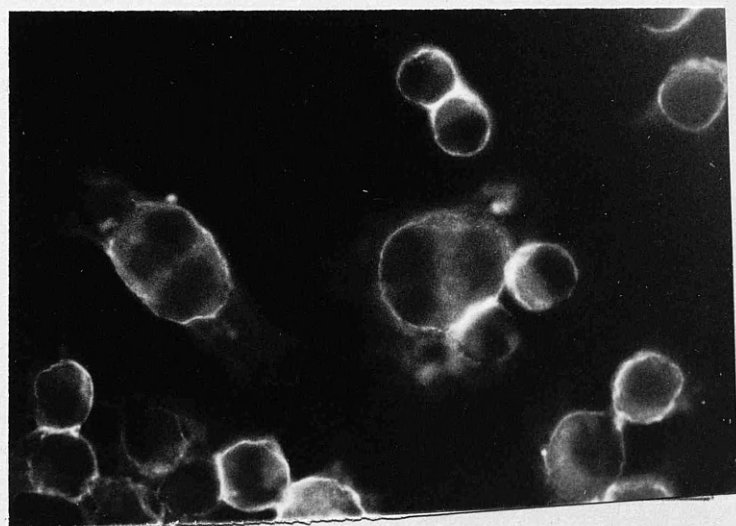
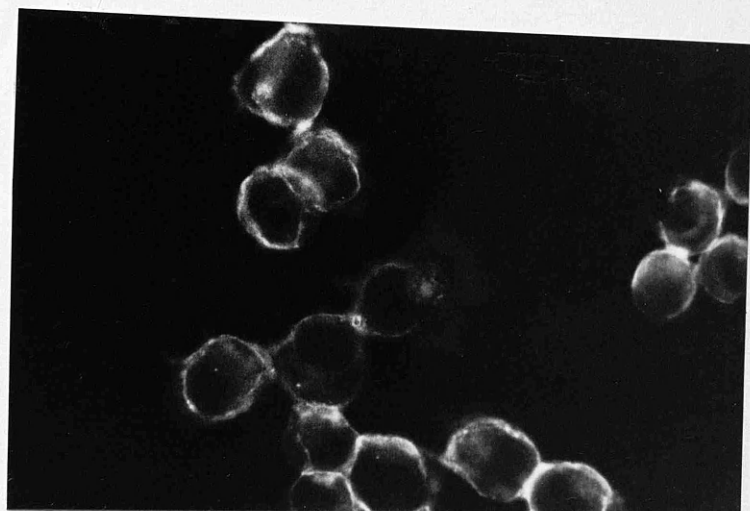


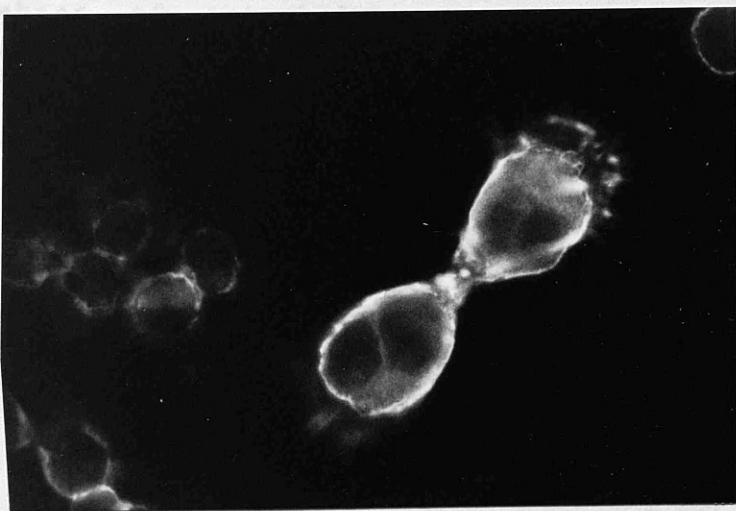
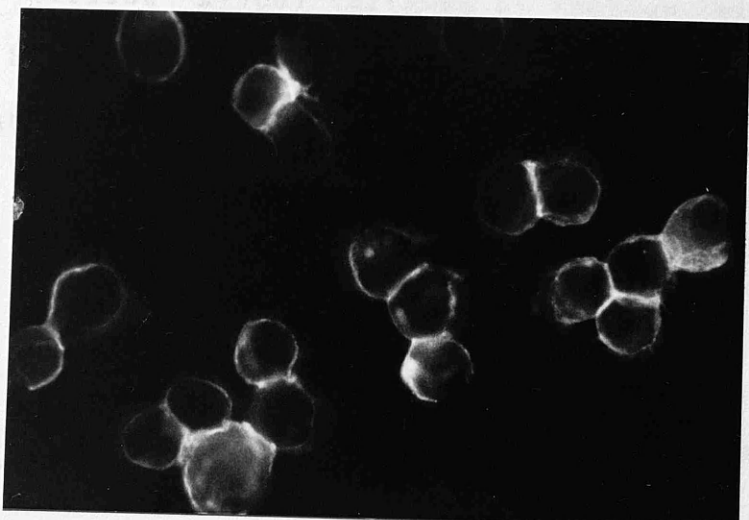
Plate 6B Variant 2

Fluorescent stain indicates the presence  
of f-actin.

Note dividing cell.

Scale Bar: 50  $\mu\text{m}$





Analysis of the neutral detergent-insoluble fraction of parental and variant cells by SDS-PAGE

Treatment of cells in vitro with a non-ionic detergent (Triton X-100) followed by various high ionic strength buffers leaves an insoluble detergent resistant residue attached to the culture dish substratum (Hynes et al., 1976). Such residues usually contain glycoproteins.

Py3 and variant cells were used in the present study using a slightly different protocol from Hynes et al. (1976) (see Materials and Methods for extraction procedure). The insoluble material obtained from the cells grown in vitro was analysed by SDS-PAGE.

Py3 cells (confluent culture) detach usually as a complete sheet of cytoskeletons in which intercellular adhesion is maintained. This is similar to the findings of Edward and Dysart (1980) on confluent cultures of BHK 21-C13 cells following extraction with non-ionic detergent. Plate (7a) shows the insoluble residues of Py3 and variant cells analyzed by SDS-PAGE gels. The bands which are indicated by arrows are actin, vimentin, nuclear material and others. Track B refers to Py3, tracks C and D refer to Var1 and Var2 respectively. Two strong 'staining bands are present in track C, one at 43 K dalton, and the other at 62 K dalton, lighter labelled bands at 75 Kd were also observed.

Plate 7A shows the insoluble residues of Py3  
and variant cells analyzed by  
SDS-PAGE gels

Track A	Standard
Track B	Py3
Track C	Variant 1
Track D	Variant 2

Arrows indicate the major common proteins  
consistently detected in both variants and Py3.  
Gel 6-10%, Coomassie stain.

Cells were washed twice in Hanks Hepes  
containing protease inhibitors (see methods) for  
15 minutes. Cells were then incubated in 1% Triton  
also containing protease inhibitor for 10 minutes.

The insoluble residue was treated with 0.1 mg/ml  
DNase in 0.5% Triton, 10 mM  $MgCl_2$  (Tris buffered)  
for 10 minutes, then with 1.5 KCl for further  
10 minutes. After a rinse with HH, the insoluble  
was solubilized in boiling mix.

Track D reveals a similar labelling pattern. Track E is 323 cells and reveals the presence of a 47 kD band and two other bands one at 42 kD and one at 74 kD dalton. Bands in the range 12-60 k dalton were also observed.

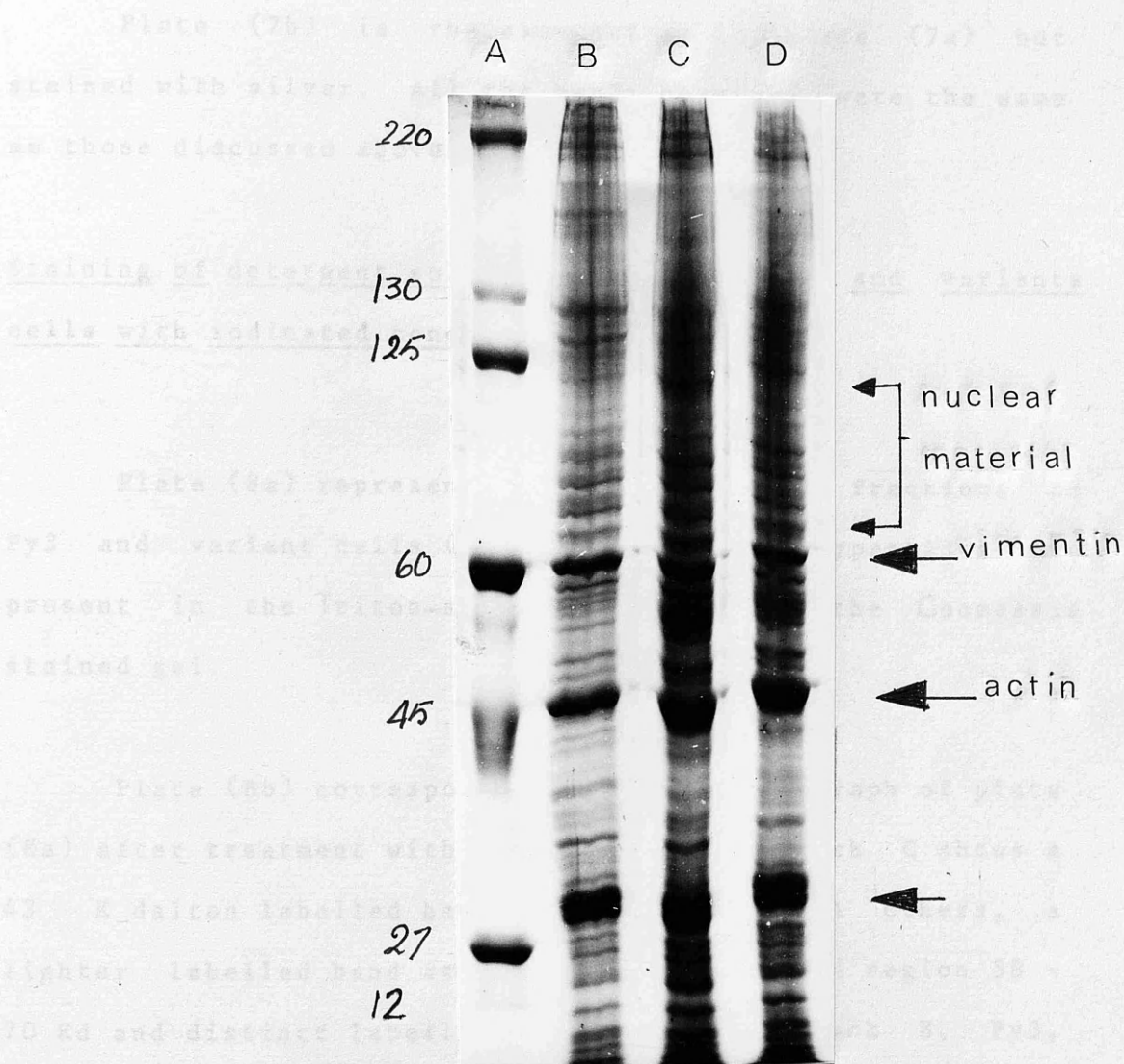


Plate (76) corresponds to the gel (75) after treatment with silver and shows the same bands as those discussed above.

Track D reveals a similar labelling pattern. Track B is Py3 cells and reveals the presence of a 43 Kd band and two other band one at 62 Kd and the second at 75 K dalton. Bands in the range 12-30 K dalton were also observed.

Plate (7b) is the same gel as in plate (7a) but stained with silver. All the bands observed, were the same as those discussed above.

Staining of detergent soluble fractions of Py3 and variants cells with iodinated concanavalin A

Plate (8a) represents the Triton-soluble fractions of Py3 and variant cells (tracks A to C). Polypeptides are present in the Triton-soluble fractions of the Coomassie stained gel.

Plate (8b) corresponds to the autoradiograph of plate (8a) after treatment with iodinated Con A. Track C shows a 43 K dalton labelled band as well as several others, a lighter labelled band at 27 Kd, wide labelled region 58 - 70 Kd and distinct labelled band at 90 Kd. Track B, Py3, track D, Var2, reveal a very similar labelled pattern.

Plate (8c) corresponds to the plate (8a) after treatment with silver staining. All three tracks revealed the same labelled pattern.

Plate 7B     Silver stained SDS-PAGE gel of  
detergent insoluble residues

Track A     Standard

Track B     Py3

Track C     Variant 1

Track D     Variant 2

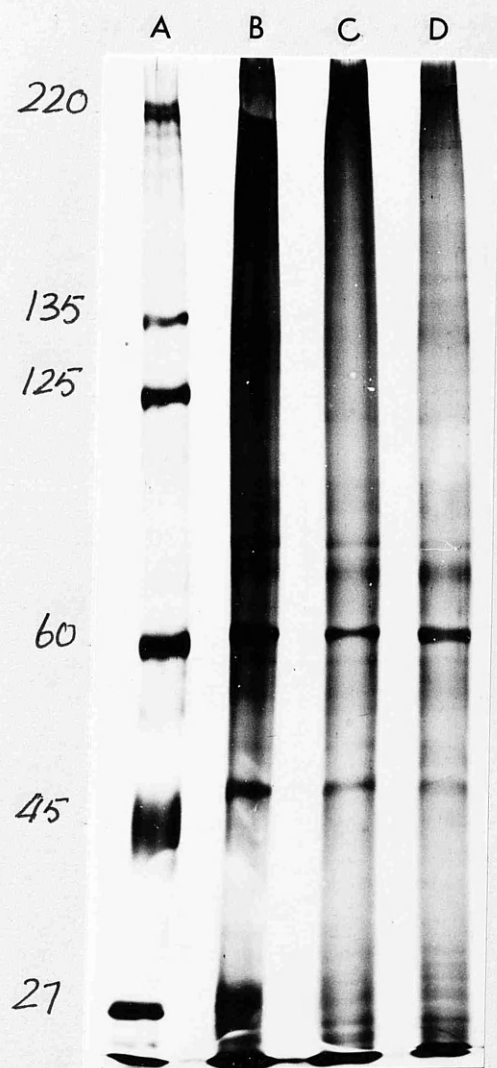


Plate 8A Shows the detergent soluble fractions  
of Py3 and variants analyzed by  
SDS-PAGE.

Track A	Py3
Track B	Variant 1
Track C	Variant 2
Track D	Standard

Arrows indicate the major proteins consistently  
detected in both variants and parental cells.



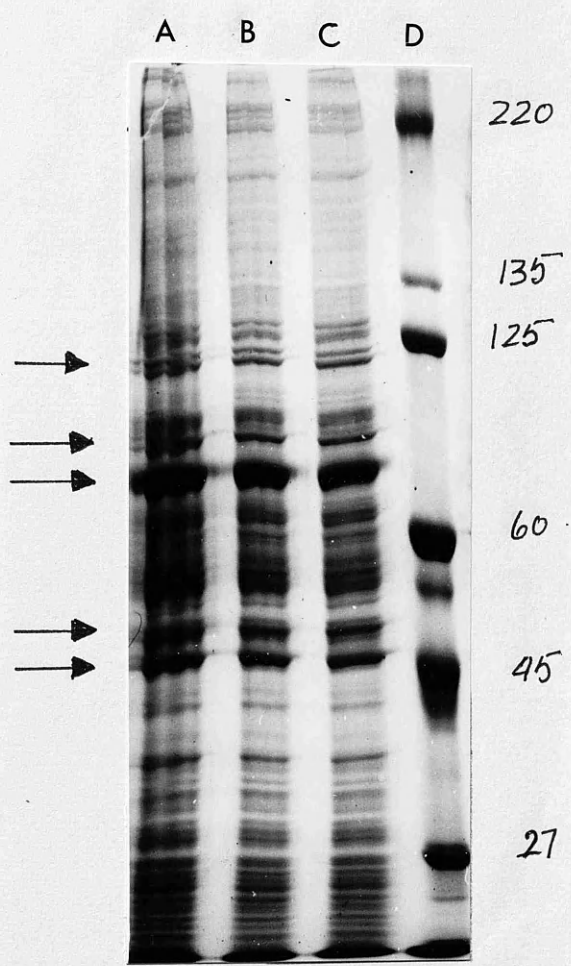


Plate 8B     Autoradiograph of <sup>125</sup>I-ConA treated  
SDS-PAGE gel of the detergent  
soluble fractions.

Track A	Standard
Track B	Py3
Track C	Variant 1
Track D	Variant 2
Track E	Standard

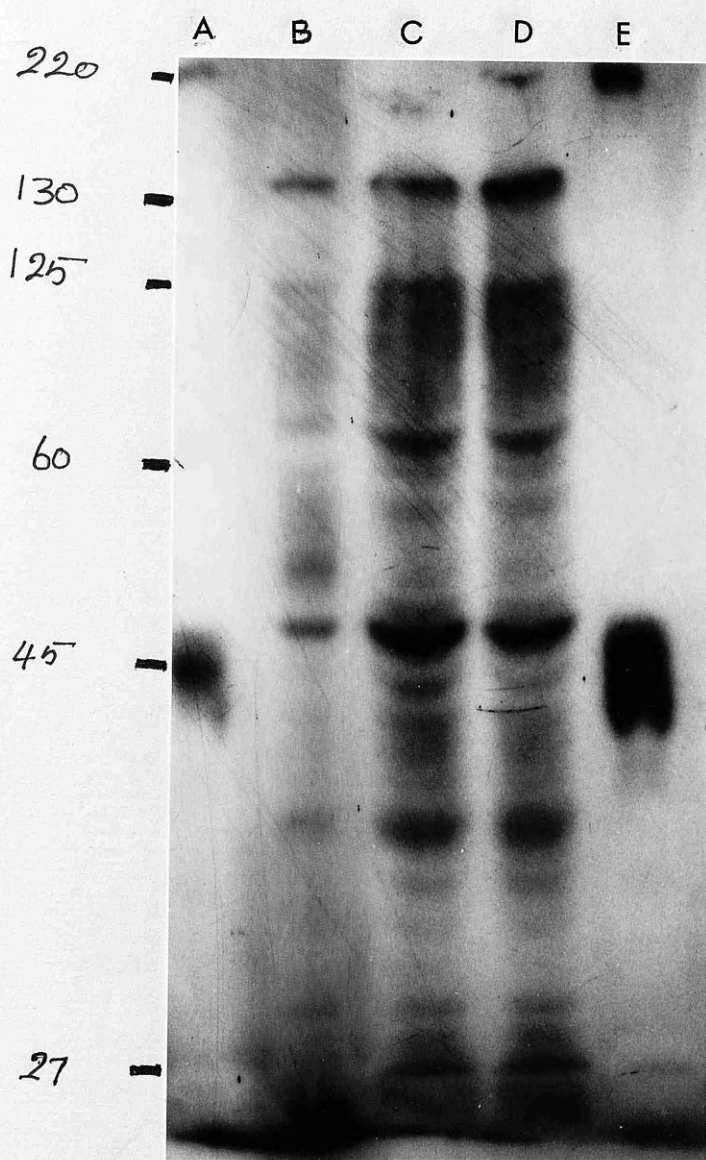


Plate 8C     Silver stained SDS-PAGE gel of  
detergent soluble fractions

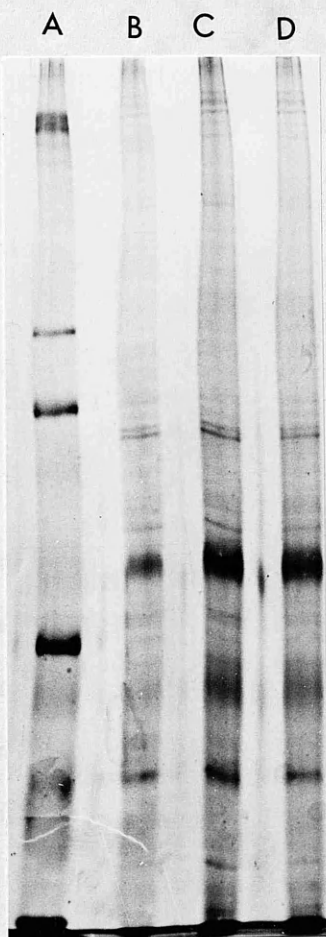
Track A	Standard
Track B	Py3
Track C	Variant 1
Track D	Variant 2

In this study, no differences in results have been observed between the two cell lines of Py3 and variant cells or between the two fractions of these cells.

### Glycopeptide analysis

In collaboration with Dr. J. H. Hill (Mill Hill), the results of fractionation of the mannose-labelled glycopeptides were analyzed. The separate analyses of the two cell lines (Py3 and the variant) showed identical results. The glycopeptides recovered from the two cell lines showed identical radioactivity applied.

Three major fractions were obtained from the column chromatography of the glycopeptides. Fraction A was eluted with buffer and showed no affinity for Con A. Fraction B was eluted with 0.1 M glucose and showed a high affinity for Con A. Fraction C was eluted with 0.2 M glucose and showed a high affinity for Con A. The results are shown in Figure 1.



The glycopeptides were further purified by gel filtration. A peak of radioactivity was observed in the fraction eluted with 0.1 M glucose, which was expected for the highly branched N-glycans. Proportions of the glycopeptides present in fraction A of Py3, variant, and control cells are shown in Table 1.

In this study, no differences in bands have been observed between detergent-insoluble residues of Py3 and variant cells or between Triton-soluble fractions of these cells.

### Glycopeptide analysis

In collaboration with Dr. R.C. Hughes (Mill Hill), the results of fractionation of [ $^3\text{H}$ ] mannose-labelled glycopeptide on concanavalin A sepharose were analyzed. Two separate analyses of the glycopeptides of each cell lines (Py3 and two variants) were carried out with essentially identical results and in all cases the radioactivity recovered from the columns represented 73-87% of the radioactivity applied.

Three major fractions were obtained. Fraction A was eluted with buffer and contained material showing no affinity for Con A. Fraction B showed weak affinity for Con A and represents bi-antennary N-glycans. Fraction C is the most highly bound fraction which contains high mannose N-glycans.

The glycopeptide fractions A were further purified by gel filtration. A peak of [ $^3\text{H}$ ] mannose radioactivity of all three cell lines was eluted from Biogel P6 in a position expected for tri, and tetra and more highly branched N-glycans. Proportions of radioactivity present in fraction A of Py3, mutant 1 and mutant 2, as estimated by Biogel P6

chromatography, were 11%, 19% and 14% respectively.

The behaviour of the high molecular weight N-glycans on Lentil lectin-sepharose was examined. Lentil lectin shows specificity for bi- or tri-antennary N-glycans that carry a fucose residue. Core fucosylated bi-antennary N-glycans show high affinity for this lectin, as do tri-antennary N-glycans carrying peripheral oligosaccharide sequences linked  $B1 \rightarrow 2$  and  $B1 \rightarrow 6$  to the core  $\alpha$ -mannosyl residues.

The radioactivity present in the fraction B, obtained from each cell line was approx. 85%, eluted from Biogel P6, and that of Lentil lectin-Sepharose columns was 70 - 74%, showed that part of the bi-antennary N-glycans of each cell line are core fucosylated.

Interestingly, the two mutant cell lines produced fractions C that appeared to be heterogenous, a secondary minor radioactive peak was eluted from Biogel P6. This peak was absent in the Py3 cell glycopeptides. The identity of this secondary peak is unknown. However, by comparison with the elution of a human glycopeptide standard (Man)<sub>5</sub>(GlcNAc)<sub>2</sub> Asn from ovalbumin, the material may be a (Man)<sub>3</sub> species, the origin of which is unknown.

In summary the proportions of radioactivity in N-acetylglucosamines that do not bind the concanavalin A, bind weakly or bind avidly are similar for each cell line.

Mutant cell lines contain a similar extent of core fucosylation as the parental cells. Mutant 2 appears to contain a significantly greater proportion of hybrid structures than either mutant 1 or parental cells.

Analysis of microsomal fractions of Py3 and variant cells by SDS-PAGE gels

Treatment of cells in vitro with Tris, sucrose and  $MgSO_4$  followed by centrifugation and resuspension in PMSF/TAME leaves sedimented unfractionated membranes (see materials and methods). This was carried out in an attempt to identify microsomal membrane proteins and also to identify the fibronectin receptor by blotting with iodinated fibronectin. This technique is established for identifying laminin receptor.

Py3 and variant cells were used in this study following protocol of Gahmberg and Simons (1970). The microsomal fraction proteins obtained from cells grown in vitro was analyzed on SDS-PAGE gels.

Plate (9) represents the pattern of the microsomal protein bands of Py3 and variant cells. No difference in bands has been observed.

In an attempt to identify fibronectin receptors of Py3 and variant cells, microsomal fraction protein bands were blotted on nitrocellulose paper ( see materials and



Plate 9. Shows the microsomal fraction  
protein of Variants and Py3  
by SDS-PAGE.

Track A	Standard
Track B	Py3
Track C	Variant 1
Track D	Variant 2

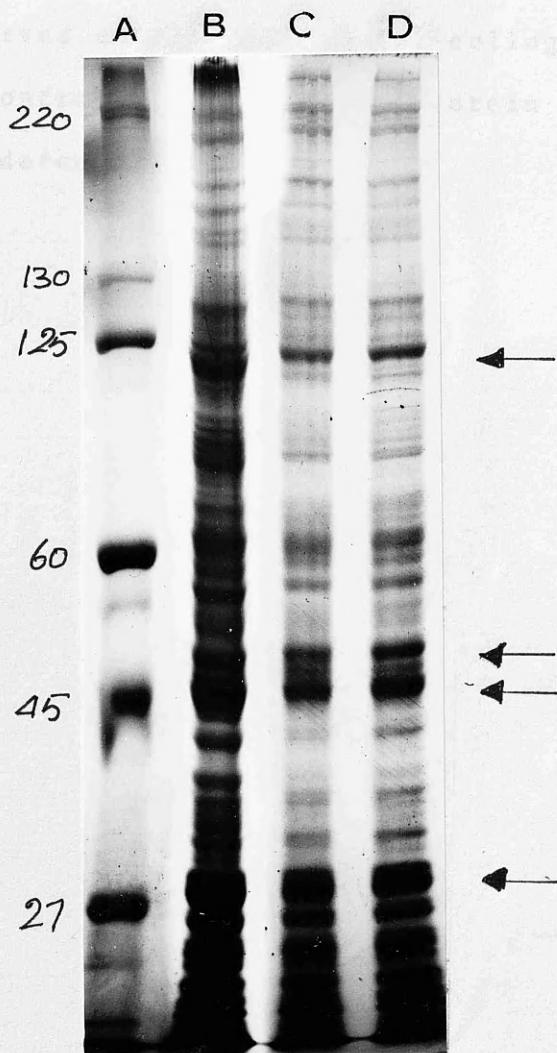
Arrows indicate the major proteins detected in  
all three cell lines.

Cells were washed twice in Tris buffered  
saline and were resuspended in TMS (see methods).  
The cell suspension was homogenized and sonicated.  
Nuclei and mitochondria were removed by centrifugation.  
The sedimented microsomal fraction was solubilized in  
boiling mix.

Gel 6-10%

Coomassie stained.

method). This technique of separation of proteins separated on SDS-PAGE gel, and detection of proteins after treatment with <sup>32</sup>P-orthophosphate by autoradiography. All proteins were detected on the nitrocellulose was done by using a haemoglobin in phosphate buffer saline. In the autoradiograph, no single band was observed.



methods). This technique produces replicas of proteins separated on SDS-PAGE gel, and allows detection of proteins after treatment with iodinated fibronectin by autoradiography. All addition binding capacity on the nitrocellulose was blocked with 0.2% haemoglobin in phosphate buffer saline. Studying the autoradiograph, no single band was observed except the one for collagen used as standard. This control showed that a protein binding fibronectin can be detected.

## Chapter Four

### DISCUSSION

The results presented here describe the selection and properties of variants of polyoma-transformed BHK-21 cells which differ from wild type Py3 cells in their ability to adhere to and spread on substrata coated with proteins such as serum or fibronectin, but which do partially spread on a clean plastic surface. These variants are unable to spread even on a surface of poly-L-lysine, to which they adhere equally with parental cells.

#### OCCURRENCE OF VARIANT

In this laboratory, BHK-21 cells transformed by two different tumour viruses have been used for selection of non-adherent variants.

1. BHK-21 cells transformed by polyoma virus (papova, a DNA virus).
2. BHK-21 cells transformed by Schmidt-Ruppin virus (a strain of avian retrovirus, an RNA virus).

Transformed cells were used because:

1. They produce almost no fibronectin (Hynes, 1976).
2. They are anchorage-independent, therefore they can grow without the need to flatten on a solid surface (Willingham et al., 1977).

These cells have a disadvantage of being poorly adherent to culture substrates. Such transformed cells might seem unpromising for the isolation of low adhesive

variants. However, their adhesion can be increased by addition of fibronectin (Yamada et al., 1977) adsorbed to the surface.

I found it was quite easy to select one mutant poorly adhesive to fibronectin from a Py3 stock after mutagenesis and equally easy to obtain another from recloned Py3 without mutagenesis. In addition, a third mutant has been obtained from a clone of Py3 selected for resistance to 6-thioguanine. Therefore these findings lead me to conclude that mutagenesis by MNNG did not noticeably help selection. So there must be a spontaneous variant arising independently at reasonably high frequency in different cloned stocks.

In this laboratory, the frequency of recovering a variant with similar properties to Schmidt-Ruppin transformed cells was zero. In a mixing experiment in which SR wild type cells were mixed with non-adherent Py-mutant at 1:10<sup>6</sup>, the Py-mutant could be recovered. Therefore, the frequency of occurrence of a SR mutant of similar property must be less than 1:10<sup>6</sup> (Hameed, unpublished work).

The differing result obtained with Py and SR cells can be interpreted by the following alternative possibilities:

1. The non-adherent phenotype may result from a mutation in polyoma DNA, coding for the T-antigens. In collaborative work with Dr. M. Fried, Imperial Cancer Research Fund, no

change in migration in SDS-PAGE of T-antigens could be detected in variants as compared with parental. No change in precipitation by a series of antipeptide monoclonal antibodies was detected. These results do not rule out a point mutation in polyoma sequences in Py3 detectable only by sequencing, but they provide no evidence for such mutation.

2. There may be cells that have different sites in cellular DNA for polyoma DNA integration, with integration at a particular site leading to the non-adherent phenotype. This could be investigated by restriction enzyme mapping.

3. There may be an altered cellular protein resulting from a mutation in cellular DNA, which together with polyoma T-antigens generates the non-adherent phenotype.

Many somatic cell lines have been selected for resistance to a variety of toxic substances (Stanley, 1980). Some variants have been selected using a single-step procedure in which the cells were exposed to selective conditions (Meager et al., 1977). Others have been isolated by a multiple enrichment procedure, in which production of the variant is a result of successive changes for resistance (Pouyssegur et al., 1977; Harper & Juliano, 1980; Klebe et al., 1977; Oppenheimer-Marks et al., 1984).

The selection used in this study is closer to a single step, there being not much growth between cycles, than to a multiple enrichment selective procedure. For comparison, in the selection of ADV (Harper &

Juliano,1980), AD6, (Pouyssegur et al.,1977), FN-1 (Oppenheimer-Marks et al.,1984), the CHO att- (Klebe et al.,1977) and hepatoma variants (Briles & Haskew, 1982), multiple selections were separated by regrowth of the cell population. However, ricin-resistant variants of BHK cells were isolated in a single step procedure (Meager et al.,1976).

#### COMPARISON WITH OTHER ADHESION-DEFECTIVE CELL LINES

Fibronectin promotes attachment and spreading of Py3 at rather low concentrations, 2 and 5  $\mu\text{g/ml}$ , but is without effect on variant cells even at concentrations as high as 25 and 50  $\mu\text{g/ml}$ . Similar findings for Adv cells were reported by Harper and Juliano (1980). However, Adv cells are capable of attaching, spreading, and attaining a normal CHO morphology on substrata coated with ligands such as concanavalin A or polylysine. Adv cells as well as wild type cells show an ability to attach and spread partially on substrata coated with SAM (substratum-attached material) (Harper & Juliano,1980). Therefore, the adhesion and spreading defect in Adv is specific to fibronectin for the surfaces tested. Cheung and Juliano (1983) also found that the CHO mutant Adv was capable of binding beads coated with plasma fibronectin as well as parental cells, but Adv cells were found not to agglutinate in the presence of beads coated with fibronectin.



Similar observations were found by Oppenheimer-Marks et al.(1984) who isolated a BHK variant FN-1 from BHK cells adapted to grow in suspension and then mutagenized with MNNG. These variant cells showed lack of spreading and low adhesion on fibronectin coated surfaces. FN-1 cells were able to attach to substrata coated with polycationic ferritin or Con A, but they did not spread (although they appeared to extend filopodia). It was also found that FN-1 cells were unable to phagocytose plasma fibronectin-coated beads. Oppenheimer-Marks et al.(1984) claim that the defect in FN-1 is specific to fibronectin. This variant is able to extend filopodia on surfaces coated with polycationic ferritin or Con A but apparently does not appear to spread normally. It is difficult to compare the variants described here with Fn-1 because there are no illustrations in the Oppenheimer-Marks report.

Briles and Haskeu (1982) described hepatoma cell variants that attach and spread normally on plasma fibronectin-coated substrates, but not on collagen films. This variant therefore may be substrate-specific. Also Klebe et al.(1977) found their mutant CHO att- to be defective in attachment to collagen coated with "cell attachment protein", presumably fibronectin. Normal morphology could be restored by 100% serum or elevated divalents (Mg increased from 1 mM to 10 mM, Ca from 1 mM to 100 mM). In addition, CHO att- cells were found to attach, but not spread, on glass and plastic surfaces.

The glycosylation defective 3T3 mutant AD6 was found to be partially spread in culture and to respond to fibronectin (Norton & Izzard, 1982). Similar partial spreading and low adhesion to substrate was also observed in ricin-resistant BHK cells (Pena & Hughes, 1978).

AD6, hepatoma cell variants and ricin-resistant BHK cells all spread on fibronectin. Of these AD6, unlike the present variants, are clearly able to spread on Con A and polylysine. FN-1 however seems very close to the variants described in this study because it could not spread normally on Con A. This can be summarized as follows:

Cell type (variant)	Substrate			
	Fn	Poly	Con A	Collagen
Adv (Harper <u>et al.</u> , 1980)	--	++	++	NT
Fn-1 (Oppenheimer-Marks <u>et al.</u> , 1984)	--	NT	ps	NT
Hepatoma variant (Briles <u>et al.</u> , 1982)	++	NT	NT	--
CHO att- (Klebe <u>et al.</u> , 1977)	--	NT	NT	NT
AD6 (Norton <u>et al.</u> , 1982)	ps	++	++	NT
Ricin resistant BHK (Pena <u>et al.</u> , 1978)	++	NT	NT	NT
Variant 1 (this work)	--	att/--	att/--	--
variant 2 (this work)	--	att/--	att/--	--

Fn, fibronectin; poly, poly-L-lysine; ++, spread; --, do not spread; att, attach; ps, partially spread; NT, not tested.

## SPREADING ON "CLEAN" SURFACE

A high proportion (35 %) of cells in a variant population showed spreading ability on a clean tissue-culture plastic polystyrene surface in serum-free medium. The proportion of variants spread is slightly less than that of the parental cells (see Fig. 9). Other workers reported that various different cell types were able to spread on clean plastic or glass surfaces. Two alternative possibilities have been suggested. First, some cells may secrete functional adhesive proteins which enable them to attach and spread on a clean surface (such as tissue culture plastic in the absence of serum). Second, there may be direct adhesion between plasma membranes and the tissue culture plastic surface. Grinnell et al. (1979) suggested that the initial attachment and spreading of human fibroblasts in serum-free medium on tissue culture plastic occurred to cell fibronectin which had been secreted and adsorbed on the substratum surface. The attachment to the substratum was prevented when protein adsorption sites on the substratum were covered with bovine serum before initial attachment of human fibroblast. Also Pizzey et al. (1984) found that cultured skin fibroblasts from normal individuals and from patients with Duchenne muscular dystrophy (DMD) spread equally rapidly and extensively when seeded on glass. The authors suggested that there was a secretion of functional adhesive proteins which enabled the cells to spread. Both DMD and normal cells were inhibited from spreading when they were preincubated with monensin (0.5  $\mu$ M). Maroudas (1977) reported that BHK cells adhered to and

spread on tissue culture dishes with or without Mn. On "sulphonated" polystyrene dishes cells were able to attach and spread in presence of Mn in serum-free medium or in the presence of serum. Curtis et al.(1983) reported that BHK and leucocyte cells preincubated with 25 µg/ ml cycloheximide to inhibit synthesis of fibronectin were able to attach and spread on chloric acid-treated polystyrene dishes in serum-free medium and the authors also found that BHK and leukocyte cells adhered to untreated polystyrene dishes with chloric acid and to oxidized polystyrene in serum-free medium. One could argue from the findings of Curtis et al.(1983) that fibronectin is not an essential component for cell attachment or spreading of BHK cells. These findings disagree with the interpretation of Grinnell et (1979) who found that fibronectin was an essential component for the spreading of fibroblasts on tissue culture grade dishes. The extent of spreading of variants is greater on a clean plastic surface than on a surface coated with fibronectin. This observation suggests that there is some direct adhesion between the plasma membrane and the tissue-culture plastic surface, which is not dependent on fibronectin.

#### EFFECT OF MANGANESE IONS

Sarcoma I cells which are non-adherent in media containing physiological levels of divalent cations such as Ca and Mg, are able to attach and spread in the presence of  $Mn^{2+}$  ions to a glass surface in the presence or absence of

serum (Rabinovitch & De Stefano, 1973). Pegrum and Maroudas (1975) reported that Mn was able to promote the attachment and spreading of BHK cells on clean polystyrene dishes, bacteriological grade, treated with sulphuric acid ( 98% from 2 minutes to 18 hours at 37 °C).

Therefore it was interesting to know the effect of Mn ions on the variants. Mn at  $10^{-4}$  M did not stimulate these variants to spread to full extent (i.e. comparable with Py3 wild type), but there was an increase in spreading in response to manganese on tissue culture plastic surfaces in absence of serum, as well as on clean glass (see Tables 2 & 5). On surfaces coated with fibronectin there was very little if any increase in cell spreading (see tables 3 & 6). These variants are unlike Sarcoma I cells in their response to Mn.

The effect of manganese on cell-substratum adhesion could be explained as follows:

1. The possibility that Mn promotes fibronectin secretion and or acts as a more efficient cofactor for fibronectin (Evans & Jones, 1982),
2.  $Mn^{2+}$  ions decrease the electrostatic force of repulsion between the cells and substrate (Weiss, 1975).
3. Mn activates a cell surface components that has receptor activity for all of the surfaces. This would account for the presence of the cell surface site prior to addition of Mn, and would also require the continued presence of Mn for activity (Grinnell, 1984).

## SURFACE-CYTOSKELETON INTERACTIONS

Stress fibres appeared very distinct in Py3 cells spread on fibronectin. F-actin bundles corresponding to filopodia were also extended by the cell during the course of spreading on fibronectin (see plate 5). The fibres are associated with the flattened appearance of Py3 and also may be correlated with the increase in substratum adhesiveness. The high concentration of fibronectin (50  $\mu\text{g/ml}$ ) in this study may be responsible for these observations as fibronectin has been reported to effect microfilament organization in cells (Ali et al., 1977).

Stress fibres were not observed in the variants in the course of this study, presumably because they did not respond to fibronectin and remained rounded. No filopodia were seen. Stress fibres were observed when wild type cells were flat. In variants, f-actin was seen underneath the plasma membrane and especially at cell-cell contact areas (see plate 6).

The interaction between cell surface molecules and the cytoskeleton was observed in the phenomenon of capping of the variant and parental cells described here. Similar observations were found by Harpér and Juliano (1980) when FITC-Con A induced capping in wild type and ADV cells. These findings suggested that the variant cells in this study may have relatively normal microfilament and microtubule systems.

Koch (1980) proposed several models to account for capping, one in particular, the anchorage model, envisages that polarized distributions of the cytoskeleton and receptors result from the attachment of surface receptors to the cytoskeleton following cross-linking, so that the receptors ultimately assume the distribution of cytoskeleton (Koch, 1980). The main drawback for the anchorage model is the inability to determine whether direct associations between the surface receptors and the cytoskeleton actually occur. The use of disruptive agents, such colchicine and cytochalasin B, only provide indirect evidence for the anchorage model.

Some glycoproteins may change the state of cytoskeletal components (recruited proteins), perhaps due to aggregation of proteins in the plane of the membrane and these glycoproteins may elicit responses involving assembly or redistribution of microfilaments. Their ability to cap shows that the variants described here can link the cytoskeletal system with Con A-binding glycoproteins in the plasma membrane.

#### STUDIES OF PROTEINS ASSOCIATED WITH TRITON INSOLUBLE MATERIAL

This present study revealed the presence of many polypeptides associated with the detergent insoluble residue of Py3 and variant cells by various techniques, but

no repeatable differences were seen (see plate 7 a). Possibly two-dimensional protein mapping could detect such a difference.

#### NATURE OF CHANGE IN THE VARIANTS

During the adhesion of cells to substrata, the initial attachment process is followed by a distinctive change in morphology resulting in a flattened and spread appearance, and the re-organization of actin filaments. Therefore, the cytoskeletal system seems to play an integral role in the change in morphology (for detail see Willingham & Pastan, 1975). The fact that both initial cell adhesion and spreading in the flow chamber was slowed by addition of cytochalasin B suggests that even initial adhesion is somewhat dependent on cytoskeletal events.

The inability of the selected variant cells to attach to and spread on any protein-coated surfaces could be due to:

1. Defects in one or more cell surface receptors which are involved in some way in the spreading response on all surfaces tested.
2. Defects in the cytoskeletal machinery involved in spreading or in its control.
3. Defects in both cell surface receptors and the cytoskeletal system (pleiotropic changes).



In the first case, the putative fibronectin receptor may be involved in some way in the spreading response on fibronectin but may also help the cells to spread on the other surfaces. In particular, when the wild type spreads on poly-L-lysine, there may be interaction between the fibronectin receptor and polylysine charge, so that the receptor induces the assembly and redistribution of microfilaments. Variants adhere, but they do not spread, perhaps due to strong non-specific bonding. Presumably in the variant there is no induction of change in cytoskeletal components such as actin.

In the flow chamber, where the cells are allowed only a very short time to adhere, very marked differences were found between variants and parental lines in adhesion to serum, fibronectin, or fibronectin-depleted serum. However all three lines adhered with equal probability to glass coated with polylysine. The variants therefore adhere well to one of these surfaces (polylysine) on which they are unable to spread.

It was found that the inhibitors cytochalasin B and EHNA decreased adhesion in the flow chamber by only 50% in comparison to untreated cells. Similar results were obtained from the static attachment assay using radiolabelled cells. Therefore, it seems that in the flow chamber at least, initial adhesion is partially independent of cytoskeletal events. The addition of inhibitors which inhibit actin polymerization slows both initial cell

adhesion and spreading. However Py3 cells still attach and in the flow chamber, in the presence of these inhibitors, whereas the mutants do not.

The inability of the variants to adhere in the flow chamber, supports the idea that there may be a defect in a receptor, so that there is no signal conveyed from fibronectin receptor to the cytoskeletal components.

The ability of the variants to cap FITC Con A would seem to indicate that different surface components involved in capping, are able to induce cytoskeletal reorganization.

## CONCLUSION

Two different interpretations of adhesive behaviour of my variants are possible:

1. There are pleiotropic changes, altering via multiple targets the initial ability of the cell-surface to bind to fibronectin (as observed in the flow chamber assay) and of the cells to spread on any surface.
2. There is a single target, such as the putative fibronectin receptor, but this receptor is also an essential component in the elicitation of spreading on the other surfaces. Non-specific surfaces such as poly-L-lysine, which induces spreading of attached cells could not recruit *inter alia*, such a specific membrane glycoprotein in the mutants.

## FUTURE INVESTIGATION

Further investigation of the genetics and biochemistry of the variants and parental cells, and selection of others under different conditions, should yield valuable information about the interplay between cell surface and cytoskeletal machinery.

Griffin et al. (1979) found that mutants (dl-8 and dl-23) of polyoma virus were due to change in polyoma DNA sequences, coding for the T-antigens, and also it has been found that the middle T-antigen is associated with cellular transformation. It is important to isolate and to determine the DNA sequence of T-antigens of variants compared with that of parental cells.

In the closing weeks of this study, a 140 K glycoprotein with properties expected of a "fibronectin receptor" was identified (Pytela et al., 1985), using its affinity for a 120 Kd chymotryptic fragment of fibronectin coupled to Sepharose. The fibronectin receptor of variant and parental cells could be investigated by this approach. Using broad spectrum antisera or monoclonal antibodies which perturb adhesion, membrane glycoproteins involved in cell-substratum adhesion could be investigated.

## Chapter Five

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